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Resistance of *Escherichia coli* to penicillins

III. AmpB, a locus affecting episomally and chromosomally mediated resistance to ampicillin and chloramphenicol

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1. INTRODUCTION

In Gram positive bacteria a step-wise increasing resistance was early found to be a characteristic for penicillin (Hotchkiss, 1951). In *Escherichia coli* K12 a step-wise increase in resistance to ampicillin was also found and a gene (*ampA*) concerned with a first step was mapped (Eriksson-Grennberg, Boman, Jansson & Thorén, 1965 and the preceding paper by Eriksson-Grennberg, 1968). *AmpA* provides resistance to a D,L-ampicillin concentration of 10–25 μg/ml. Strains carrying *ampA* mutate spontaneously to become resistant to a D,L-ampicillin concentration of 50 μg/ml. Such double mutants carry a second mutated gene (*ampB*), which is known to be distant from *ampA*. In this communication we report the construction of a strain assumed to carry only the *ampB* locus. This strain and others have been used to study the effect of chromosomal mutations on the resistance mediated by R-factors. It was found that *ampB* enhances the effects of both episomal and chromosomal genes for ampicillin and chloramphenicol resistance. Preliminary accounts of the work have been reported at meetings (Boman, Eriksson-Grennberg, Földes & Lindström, 1967; Boman, Nordström, Eriksson-Grennberg & Normark, 1968).

2. MATERIALS AND METHODS

(i) *Organisms*

All strains used were derivatives of *E.coli* K 12 their properties and origins are shown in Table 1.

The two R-factors used were R1, which carried resistance to ampicillin, chloramphenicol, kanamycin and sulphonamide, and R_{TEM}, which carries resistance to ampicillin and streptomycin. Both of the R-factors and the carrier strain RC711 have been described by Meynell & Datta (1966).

Phage P1bt described by Gross & Englesberg (1959) was obtained from Dr R. Helling.

(ii) *Media*

Minimal medium was made from the basal medium E of Vogel & Bonner (1956) supplemented with 0·2% glucose, 1 μg/ml of thiamine, the required amino acids

Table 1. Escherichia coli *K12 strains used and their relevant characters*

Strain	Origin	Sex*	Ampicillin resistance: Genotype: Site 1	Ampicillin resistance: Genotype: Site 2	Ampicillin resistance: Phenotype†	Other relevant markers‡	Response to str
G11	Stent & Brenner, 1961	Hfr	+	+	amp-s	*ilv*, *metB*	s
G11b4	Eriksson-Grennberg *et al.* 1965	Hfr	+	*ampB2*	amp-2	*ilv*, *metB*	s
G11a1	Eriksson-Grennberg *et al.* 1965	Hfr	*ampA1*	+	amp-10	*ilv*, *metB*	s
G11e1	Eriksson-Grennberg *et al.* 1965	Hfr	*ampA1*	*ampB1*	amp-20	*ilv*, *metB*	s
Hfr Reeves 1	Obtained from R. Hill	Hfr	+	+	amp-s	*metB*	s
R12	See Fig. 1	Hfr	*ampA1*	+	amp-s	*metB*	s
PA256	Obtained from R. Lavallé	F⁻	+	+	amp-s	*pro*, *his*, *arg*, *purA*	r
KG20	See Fig. 1 and text	F+	*ampA1*	+	amp-1	*pro*, *his*, *arg*, *purA*	r
KG20e1	See Fig. 1 and text	F+	*ampA1*	*ampB3*	amp-20	*pro*, *his*, *arg*, *purA*	r
KG21	See Fig. 1 and text	F+	+	*ampB3*	amp-2	*pro*, *his*, *arg*,	r
KG29	See Fig. 1 and text	?	+	+	amp-s	*pro*, *his*, *arg*,	r

* The presence of *F*-factor was assumed from sensitivity to the RNA phage MS2. For KG20, KG20e1 and KG21 plaques were obtained with 0·2 ml culture and overnight incubation at 37 °C.

† The phenotypes are denoted amp-s for the wild type and amp-n for the mutants when *n* is defined as the ratio between the resistance of the mutant and the resistance of the wild type.

‡ Abbreviations: amp, ampicillin; arg, arginine; his, histidine; ilv, isoleucine-valine; met, methionine; pro, proline; pur, purine; str, streptomycin, r = resistance, s = sensitivity. The capital letters after some of the symbols refer to the nomenclature of Taylor & Trotter (1967).

(25 μg/ml of the L epimer), and in some cases adenine (10 μg/ml). Plates were solidified with 1·5% agar.

For strain PA256 and descendants, it was necessary to supplement the minimal medium with a solution of eleven amino acids and with vitamins (see Eriksson-Greenberg, 1968).

The complete medium was the LB of Bertani (1951) (with, however, 0·2% glucose) supplemented with the minimal medium E of Vogel and Bonner (1956) and 1 μg/ml of thiamine. Plates with complete medium (LA) contained LB supplemented with $2{\cdot}5 \times 10^{-3}$M-$CaCl_2$, 1% of vitamin solution (see Eriksson-Grennberg, 1968) and were solidified with 1·5% agar.

(iii) *Materials*

The following compounds were kindly donated to us: Ampicillin (α-amino benzyl penicillin) with D- and L-epimers in the ratio 4:6 and the pure D-epimer by Astra, Södertälje, Sweden; chloramphenicol as the free base and streptomycin sulphate by Kabi, Stockholm, Sweden. Nylon microculture containers used for replica plating were obtained from Elesa, Milano, Italy.

(iv) *Mating conditions*

These were as described by Eriksson-Grennberg *et al.* (1965). However, LB was used as conjugation medium, since PA256 and its descendants do not grow in the medium previously used.

(v) *Transfer of R-factors*

The following procedure was used to avoid any selection for ampicillin resistance. Diluted overnight cultures in LB were grown logarithmically at 37° to a cell density of about 1×10^8 per ml (measured turbidimetrically). 10 ml of the recipient culture was mixed with 1 ml of the donor culture (RC711 with R1 or R_{TEM}) and incubated at 37° without shaking. The transfer was interrupted after 60 min by blending, but incubation was continued on a rotary shaker for 2 h more to allow for phenotypic expression of the resistance. Aliquots were diluted in 0·9% NaCl and spread on minimal medium supplemented to select for resistance and to allow the recipient but not the donor cells to grow. When R1 was transferred plates contained 100 μg of chloramphenicol/ml, for R_{TEM} 10 μg of streptomycin/ml was used. Colonies were picked after about 40 h at 37°, and restreaked on the same medium. They were tested for markers and for ampicillin resistance. The transfer frequency per donor cell was of the order 10^{-3} to 10^{-4}. Since PA256 and its descendants are streptomycin-resistant, the technique described above could not be used for the transfer of R_{TEM}. In these cases streptomycin was replaced by 100 μg/ml of ampicillin.

(vi) *Transduction experiments and test for immunity to P1bt*

The procedure used for transduction is described in detail by Eriksson-Grennberg (1968). To test for immunity loops of phage P1bt at different dilutions were spotted on soft agar containing 0·05–0·1 ml of a full-grown culture of the bacteria to be

tested. The plates were incubated at 37° overnight. Clearing at 10^8 plaque-forming units (PFU)/ml was taken as evidence of sensitivity. Immune strains did not show clearing at 10^{10} PFU/ml.

(vii) *Determination of resistance*

For strains without R-factors preliminary tests were carried out with replica plating (cf. Materials) on LA plates with the ampicillin concentrations 4, 10, 50 and 100 μg/ml. Due to the high penicillinase activity mediated by R-factors this technique gave too high estimates of the resistance, unless the inocula were diluted to less than 100 cells per spot. For preliminary tests of strains with R1, concentrations of 0, 100, 200, 300, 400 and 500 μg/ml of D,L-ampicillin were used and for R_{TEM} 0, 600, 1000, 1500, 2000 and 3000 μg/ml. The final determination of resistance in both cases was based on ability to form single cell colonies on ampicillin plates. The resistance level is given as the highest concentration at which all cells plated gave rise to colonies when incubated overnight at 37 °C. PA256 and derivatives grow slowly and were incubated for about 40 h. In each experiment reference cultures were included as biological standards.

Since chloramphenicol does not kill the bacteria but retards their growth, tests for chloramphenicol resistance were more difficult to read than were penicillin tests. At certain concentrations the colonies were smaller than on the control plates and the result was therefore dependent upon the time when the plates were read. We have defined the resistance level as the highest concentration at which all cells plated gave rise to colonies of normal size and read the plates as soon as the control plate showed normal colony size.

3. RESULTS

(i) *Determination of ampicillin resistance*

Resistance was determined by plating about 200 cells on LA plates with increasing concentrations of ampicillin. Fig. 1 shows an example of the results obtained with four different strains and the D,L-epimers (4:6) as well as the pure D-epimer of ampicillin. The resistance level was defined as the highest concentration at which the colony count was the same as in the control. For all strains a 50% increase in this concentration reduced the colony count to 0 (the curves of Fig. 1 are essentially parallel). The relative resistance of different strains was quite reproducible. However, the D- and L-epimers are not equally effective as antibiotics and both are somewhat unstable. For these reasons the absolute resistance levels varied somewhat from experiment to experiment. It was therefore found necessary to include well-known strains as biological standards in each determination. On plates with reduced colony counts, the colonies were smaller than on the control plates.

Despite the indicated limitations it was found that single-cell colony formation was drastically affected within a rather narrow concentration range. Thus it was easy to distinguish strains whose resistance differed by less than a factor of two.

(ii) *Genetic separation of the* ampB *locus from* ampA

We have previously described two Hfr strains resistant to D,L-ampicillin concentrations of 10 μg/ml which carry the *ampA* gene localized between *argH** and *pyrB* (Eriksson-Grennberg *et al.* 1965). More resistant strains were assumed to be double mutants carrying, in addition to *ampA*, a second gene (*ampB*). However, we failed to transfer the higher resistance with derivatives of Hfr Cavalli and

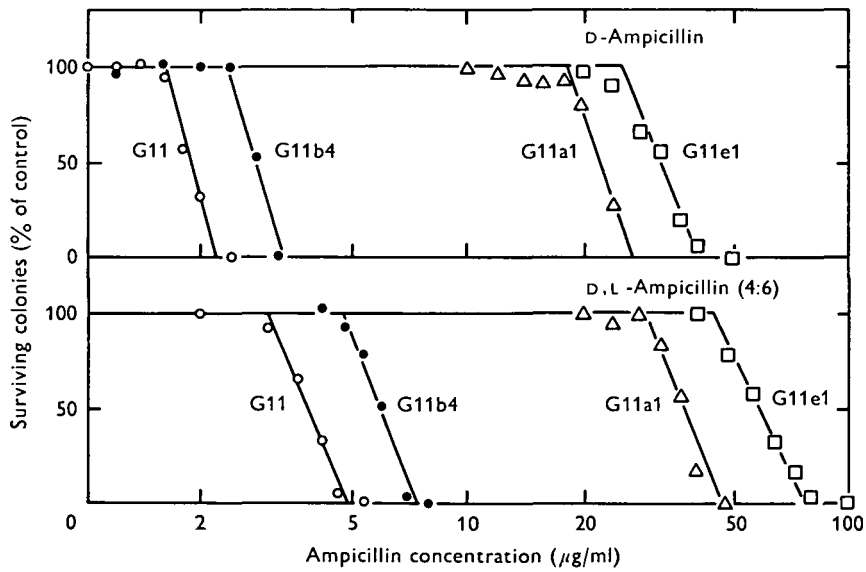

Fig. 1. Resistance of the G11 strains with different combinations of the *ampA* and *ampB* genes, estimated as the ability to form single cell colonies on plates with different concentrations of D-ampicillin (upper diagram) and D,L-ampicillin (ratio 4:6) (lower diagram).

P4X. Nonetheless some properties of the *ampB* mutation could be studied in a strain (KG21) which carries this mutation alone. This strain was constructed utilizing the cotransduction between *ampA* and *purA* described by Eriksson-Grennberg (1968). By an analogous cotransduction a strain (KG29) was obtained which contains the wild-type alleles of both the ampicillin genes. KG21 and KG29 and their ancestors KG20 and KG20e1 represent a set of essentially isogenic strains, differing only in the *ampA* and *ampB* genes. A summary of the genetic steps involved in the construction of these strains is given in Fig. 2 in the form of a pedigree. The four KG strains are all sensitive to P1bt, showing that they are not lysogenic for P1. The male-specific RNA phage MS2 produced plaques on KG20, KG20e1 and KG21 but no ton PA256 or KG29. The MS2-sensitive strains may have received the F-factor from the R12 culture used (see Fig. 2), since this strain has been observed to revert from the Hfr to the F^+ form.

We have previously failed to obtain any spontaneous one-step mutants or

* Nomenclature of Taylor & Trotter (1967)—previously designated *argF*.

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tranductants resistant to D,L-ampicillin concentrations of 50 μg/ml (Eriksson-Grennberg *et al.* 1965). However, a strain which already carries the *ampB* locus would be expected to give the amp-20 phenotype in one step (for phenotypes cf. Table 2 and discussion, part i). This was confirmed by transducing the *ampA* gene

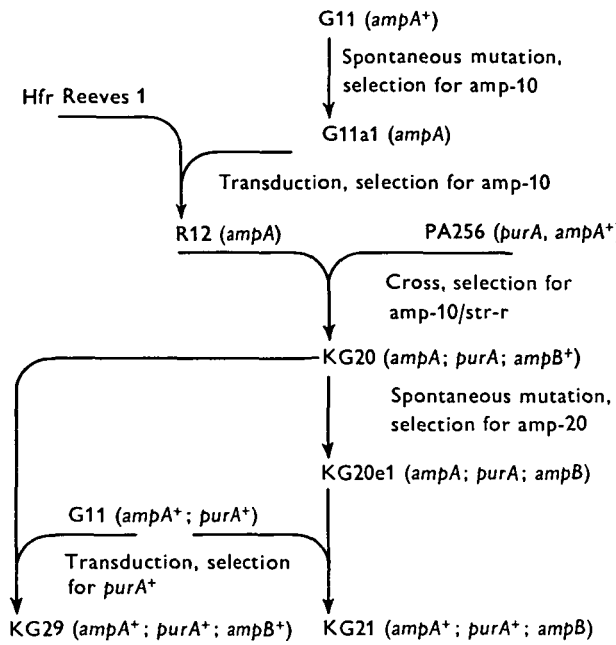

Fig. 2. Pedigree for strains KG20, KG20e1, KG21 and KG29. After the strain denotation, the relevant genes are given within parentheses.

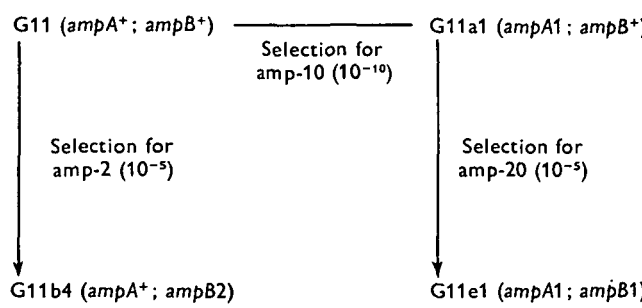

Fig. 3. Pedigree for strains G11, G11b4, G11a1 and G11e1. All mutants were spontaneous. The ampicillin genotypes are given after the strain denotations. The mutation frequency is given within parentheses.

from G11a1 into strain KG21. Selection was done on plates with 10 and with 50 μg of D,L-ampicillin/ml. Colonies were obtained on both types of plates. Those from plates with 10 μg/ml of ampicillin were picked and tested; all were found to be of the amp-20 phenotype. It was also possible to mutate KG21 to amp-20 in one step.

Table 2. *The effect of* ampA *and* ampB *on R-factor mediated resistance*

	Ampicillin resistance							
				Resistance level (μg/ml)*				
	Genotype			No R-factor		With R1		
Strain	Site 1	Site 2	Phenotype†	D-ampicillin	D,L-ampicillin‡	D-ampicillin	D,L-ampicillin‡	With R_{TEM} D,L-ampicillin‡
G11	+	+	amp-s	1	4	75	225§	700
G11b4	+	*ampB2*	amp-2	2	6	150	300–400	1500
G11a1	*ampA1*	+	amp-10	15	25	75	250§	700
G11e1	*ampA1*	*ampB1*	amp-20	25	50	200	400–500	1500
PA256	+	+	amp-s	0·5	2	75	150	700
KG29	+	+	amp-s	0·5	2	50	100	700
KG21	+	*ampB3*	amp-2	1	4	150	300	150
KG20	*ampA1*	+	amp-10	6	10	50	150	700
KG20e1	*ampA1*	*ampB3*	amp-20	15	25	150	300	1500

* Resistance is given as the highest concentration of ampicillin (in μg/ml) at which all cells plated gave rise to colonies on LA plates. The following concentrations of ampicillin were used: 0, 0·5, 1, 2, 4, 6, 8, 10, 15, 25, 50, 75, 100, 150, 200, 300, 400, 500, 700, 1000, 1500 and 2000 μg/ml.

† Cf. footnote Table 1.

‡ D- and L-epimers in the ratio 4:6.

§ For these two strains plates with 225, 250 and 275 μg/ml. of ampicillin were included in the resistance test.

Strains G11, G11a1 and G11e1 have been described by Eriksson-Grennberg *et al.* (1965). Their inter-relationship is given in Fig. 3. There are reasons to assume that G11b4 differs from G11 by a mutation in the *ampB* gene. G11b4 was isolated in the same way as G11b1 described by Eriksson-Grennberg *et al.* (1965). The spontaneous mutation frequency was the same for G 11 → G11b4 and for G11a1 → G11e1. Further evidence for the presence of *ampB* in G11b4 is given in the next section.

The resistance of the strains described was estimated as the ability to form single cell colonies on plates containing various amounts of ampicillin. Table 2 shows that the *ampB* gene in G11b4 and KG21 doubled the resistance of the wild type strains G11 and KG 29. Also in the *ampA* strains G11a1 and KG20 the presence of *ampB* provided a two-fold increase in resistance.

(iii) *Effects of chromosomal genes on R-factor mediated ampicillin resistance*

Episomes providing penicillin resistance in *E. coli* are known to mediate an extensive production of penicillinase (see review by Datta, 1965). In order to study the effect of the chromosomal genes on the R-factor mediated resistance, the episomes R1 (Meynell & Datta, 1966) and R_{TEM} (Datta & Richmond, 1966) were transferred to strains carrying different combinations of *ampA* and *ampB*. The resistance of these strains without and with one and the other of the episomes is given in Table 2. The results show that *ampA* increased the resistance provided by R1 only slightly, while the effect of *ampA* and *ampB* or of *ampB* alone was an increase of the order of 100–200%. R1 mediated the same resistance in G11b4 and KG21 which is consistent with the presence of *ampB* in both strains. The resistance provided by R_{TEM} was almost too high to be measured by the procedure used and the figures obtained with this episome are therefore not very accurate. However, the tendency shown in Table 2 is that the effects of *ampA*, R1 and R_{TEM} were additive, while *ampB*, increased the effects of the other genes by two-fold or more.

Table 3. *Effect of* ampA *and* ampB *on chloramphenicol resistance*

	Ampicillin genes		Chloramphenicol resistance (μg/ml)*	
Strains	Site 1	Site 2	No R-factor	With R1
G11	+	+	4	300
G11b4	+	*ampB2*	6	600
G11a1	*ampA1*	+	3	200
G11e1	*ampA1*	*ampB1*	6	600
KG29	+	+	0·5	150
KG21	+	*ampB3*	1·5	300
KG20	*ampA1*	+	1·0	150
KG20e1	*ampA1*	*ampB3*	1·0	300

* Single cell test on LA plates. Concentrations of chloramphenicol tested: 0, 0·5, 1, 1·5, 2, 3, 4, 6, 8, 100, 150, 200, 300, 400, 600 and 800 μg/ml.

(iv) *Effects of* ampB *on resistance to chloramphenicol*

Chloramphenicol resistance was determined by single-cell-colony formation on LA plates with different concentrations of chloramphenicol. Table 3 shows that R1 mediates rather high resistance to chloramphenicol. A comparison of the strains which carry *ampB* shows that this mutation doubles the chloramphenicol resistance of R1. The strains used have the same level of resistance as found by Reeve (1966) for the wild type genes. Our result in Table 3 would therefore indicate that *ampB* can enhance also the wild-type chromosomal resistance. If *ampA* has any effect it seems to mediate a minor decrease in the chloramphenicol resistance.

DISCUSSION

(i) *The method for determining resistance*

We have previously used ampicillin with the D- and L-epimers in a ratio of 6:4, while the present batch had the ratio 4:6. It is difficult to get ampicillin of a high degree of purity and with a reproducible epimer ratio. Since the D-epimer gives faster lysis than does the L-epimer (Boman & Eriksson, 1963), the result of resistance determinations clearly depends on the epimer ratio. Furthermore, the L-epimer is less stable than the D-epimer (Sjöberg & Östergren, Astra, Sweden, personal communication). Thus we have decided to use only the pure D-epimer in future work. To facilitate comparison with earlier data we have included results obtained with both the D,L- and the pure D-epimers of ampicillin (Fig. 1 and Table 2).

Provided that biological standards are included, resistance determinations by single cell colony formation on different concentrations of ampicillin was found to be a relatively reliable and accurate method. A two-fold difference was considered clearly significant and similar accuracy was found by Reeve (1966) for chloramphenicol resistance.

Table 2 shows that the G11 strains are more resistant than the corresponding derivatives of PA256. The precise resistance level mediated by *ampA* or *ampB* therefore depends on the strains used. This creates difficulties in defining the phenotypes. Table 2 shows that *ampA* and *ampB* each increase resistance by a constant and characteristic factor of about 10 and 2 respectively. These factors were therefore used to define the phenotypes (cf. Tables 1 and 2).

(ii) *The effect of the* ampB *locus on ampicillin resistance*

The *ampA* gene is rather well mapped (Eriksson-Grennberg, 1968). A penicillinase has been purified from two *ampA*-containing strains as well as from the wild type (Lindström & Boman, 1968). Datta & Richmond (1966) have purified the penicillinase mediated by R_{TEM} and the corresponding R1 enzyme is at present under purification (Lindqvist & Nordström, to be published). Preliminary comparisons show that all three enzymes differ both in molecular weight and certain properties. Since both *ampA* and the two R-factors mediate penicillinases it is

possible to interpret the additive figures for resistance in Table 2 as the summation of different penicillinase effects.

We have earlier suggested that in the double mutant G11e1 part of the resistance could be due to amidase activity (Eriksson-Grennberg *et al.* 1965). To test this possibility we have used a semi-synthetic penicillin with methionine as side chain and in the absence of methionine tried to select amidase mutants from a methionine-requiring strain. No colonies grew on this penicillin or on the corresponding penicilloic acid obtained by treatment with penicillinase. We have also shown that an amidase-producing strain (obtained from Astra) was as sensitive as our most sensitive strains. These results would seem to rule out amidase as a source of resistance in our *ampB*-containing strains.

Table 2 shows that *ampB* provides a two- to three-fold increase in the resistance mediated by the chromosomal *ampA* gene or by the two episomes R1 and R_{TEM}. The effect resembles that of the modifying genes which Ahmed & Woods (1967) have found during analysis of nystatin resistance in *Saccharomyces cerevisiae*. The fact that *ampB* doubled the chloramphenicol resistance of both R1 and the chromosomal genes indicates that *ampB* has a modifying function also on chloramphenicol resistance. It is interesting that Reeve (1966), using R1, has found two types of genes for chloramphenicol resistance, one of which increases the R-factor mediated resistance to a considerable extent. The results in Tables 2 and 3 also suggest that R-factors can be useful for the identification of modifying genes which together with wild type genes provide only a minor increase in resistance.

A number of observations indicate that *ampB* is a pleotropic locus. Besides the effects described here it was previously found that *ampB* reduced the growth rate-dependent adjustment both of the *ampA* penicillinase and the time to lysis. It was also observed that some *ampB*-containing mutants had lost the ability to use low concentrations of succinate as sole carbon source (Boman *et al.* 1967). In mating experiments some *ampB*-containing strains were also found to produce delayed transfer of the chromosome (Boman, Eriksson-Grennberg, Normark & Matsson 1968). Recently Nordström & Burman (1968) have found that *ampB* gives an increased sensitivity to osmotic shocks and to cycloserine. Probably as a consequence of *amp*B, some normally cell bound enzymes leak out into the growth medium. All these effects seem to be consistent with the hypothesis that both *ampA* and *ampB* affect cell wall synthesis. However, we have still no evidence concerning the nature of the gene product of the *ampB* locus.

SUMMARY

We have previously described strains of *Escherichia coli* K12 resistant to D,L-ampicillin concentrations of 50 μg/ml (Eriksson-Grennberg *et al.* 1965; Boman *et al.* 1967). Such strains were assumed to be double mutants carrying the genes *ampA* and *ampB*. We here describe genetic steps which produce strains carrying only the *ampB* gene. Determinations of resistance showed that *ampA* increased

the resistance provided by *ampA*$^+$ by a factor of 10–15. *AmpB* increased the resistance of both *ampA* and *ampA*$^+$ by a factor of 2.

R-factors were introduced into two sets of strains with all combinations of *ampA*, *ampB* and their wild type alleles. *AmpA* and the R-factors gave additive effects on resistance, while *ampB* doubled the ampicillin resistance mediated by *ampA* as well as by the R-factors. *AmpB* also enhanced the chloramphenicol resistance of R-factor and of the wild type chromosomal genes. It is suggested that *ampB* resembles the modifying genes previously described and that R-factors can be useful for the identification of such genes.

We wish to express our thanks to Drs G. Bertani, E. C. R. Reeve and B. Sjöberg for several helpful discussions. Drs N. Datta, R. Lavallé and R. Hill kindly provided some of the strains used. Dr B. Sjöberg and his colleagues at Astra kindly synthetized the methionyl penicillin and provided the amidase-producing strain. The technical assistance of Miss Britt Hansson is gratefully acknowledged. The work was supported by grants from the Swedish Natural Science Research Council (Dnr 2453) and from the Swedish Cancer Society (Nr. 68:44).

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