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Resistance of *Escherichia coli* to Penicillins

I. Genetic Study of Some Ampicillin-Resistant Mutants

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ABSTRACT

ERIKSSON-GRENNBERG, KERSTIN G. (University of Uppsala, Uppsala, Sweden), HANS G. BOMAN, J. A. TORBJÖRN JANSSON, AND SONE THORÉN. Resistance of *Escherichia coli* to penicillins. I. Genetic study of some ampicillin-resistant mutants. J. Bacteriol. **90**:54–62. 1965.—A number of ampicillin-resistant mutants have been isolated and characterized. Of these strains, two groups have been genetically investigated: members of one group, which are moderately resistant to ampicillin (Amp^r_{10}) carry mutations in a locus which we have designated *ampA*; another strain, which is resistant to high levels (50 μg/ml) of ampicillin, is a multistep mutant for which a genotype cannot yet be written. The phenotype of this strain has been designated Amp^r_{50}. The location of *ampA* was studied by the interrupted-conjugation technique, with *argF*, *metB*, *mtl*, and *serA* as reference markers. The phenotypic expression was the same for *ampA* and *argF*. These experiments, as well as recombination without selection for ampicillin resistance, indicate that *ampA* is located between *argF* and *pyrB*. "Broken slopes" on the recombinant curves and failure to demonstrate cotransduction make a more accurate mapping difficult. Phage P1bt transduced *ampA* from resistant donors to sensitive recipient strains with a frequency of 5×10^{-7}. The *ampA* locus segregated in conjugation and transduction experiments with an Amp^r_{50} donor strain, but neither method gave a genetic transfer of high resistance to ampicillin. Penicillinase activity was demonstrated in two independent mutants carrying the *ampA* locus.

The development of penicillin resistance in bacteria was early found to be a multistep process involving several consecutive mutations and giving rise to clones with gradually increasing penicillin resistance (*see*, e.g., Demerec, 1945; Hotchkiss, 1951; Cavalli and Maccacaro, 1952; Banič, 1959). In most bacteria examined, the cause of penicillin resistance has been attributed to the production of enzymes which inactivate penicillin. Two such enzymes are known: penicillinase (β-lactamase), which hydrolyzes the CO—N bond in the β-lactam ring of the penicillin molecule (Pollock, 1960, 1962), and amidase, which hydrolyzes the CO—NH bond between the side chain and the 6-amino group in the penicillanic acid residue (English, McBride, and Huang, 1960). Both of these enzymes are known to exist in *Escherichia coli*, but only a few studies of their properties have been published (Percival, Brumfitt, and de Louvois, 1963; Holt and Stewart, 1964*a*, *b*).

The detailed knowledge on penicillinase from *Bacillus cereus* (Pollock, 1960, 1962) has so far been difficult to correlate with a genetic analysis. We therefore planned a genetic study of penicillin resistance in *E. coli*, where a wealth of genetic data was available (Hayes, 1964). The fact that several thousands of penicillin derivatives have been synthesized (Abraham and Newton, 1961) should provide a good opportunity for the selection of mutants affected at the active site(s) of penicillin binding. We have in a previous paper compared the rate of lysis obtained with penicillins having 14 different side chains (Boman and Eriksson, 1963).

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MATERIALS AND METHODS

Organisms. All strains used were *E. coli* K-12; Table 1 shows their properties. Strains AB325 (λL26) and G11 were obtained from G. Stent; G11 was described by Stent and Brenner (1961). AB325 was described by Taylor and Adelberg (1960). Selection conditions used for the isolation of a series of spontaneous ampicillin-resistant mutants of G11 are given in Table 2. P4xa2 is a one-step ampicillin-resistant mutant of strain P4x (Adelberg and Burns, 1960). P4xa21 was obtained from P4xa2 by transducing it from Met^- to Met^+ with P1bt grown on *E. coli* B. KG3 is an

TABLE 1. *List of Escherichia coli K-12 strains and markers used**

Strain	Auxotrophic characters								Response to str	Amp. permissible in plates	Genotype assigned for amp response	Sex
	his	*serA*	*mal*	*xyl*	*mtl*	*ilva*	*metB*	*argF*				
										μg/ml		
AB325(λL26)....	−	−	−	−	−	+	+	−	r	<1	*amp*+	F−
KG3.....	−	+	−	−	−	+	+	−	r	10	*ampA2*	F−
KG110(P1).....	−	+	−	+	+	+	−	−	r	<1	*amp*+	F−
P4x.....	+	+	+	+	+	+	−	+	s	<1	*amp*+	Hfr
P4xa2.....	+	+	+	+	+	+	−	+	s	10	*ampA2*	Hfr
P4xa21.....	+	+	+	+	+	+	+	+	s	10	*ampA2*	Hfr
G11.....	+	+	+	+	+	−	−	+	s	<1	*amp*+	Hfr
G11a1.....	+	+	+	+	+	−	−	+	s	10	*ampA1*	Hfr
G11e1.....	+	+	+	+	+	−	−	+	s	50	(unknown)	Hfr

* Abbreviations used: amp, ampicillin; arg, arginine; ser, serine; his, histidine; ilva, isoleucine and valine; lac, lactose; mal, maltose; met, methionine; mtl, mannitol; (*o*), origin of chromosome; pro, proline; pyr, pyrimidine; str, streptomycin; thr, threonine; xyl, xylose; s, sensitive; r, resistant. P4x transfers chromosomal markers in the order: (*o*)-*pro-ilva-serA*; G11 in the order (*o*)-*lac-pro-ilva*.

ampicillin-resistant recombinant, and KG110 an ampicillin-sensitive recombinant, both selected as *serA*+ *str-r* from a cross of P4xa2 × AB325 (λL26). Derivatives of P4x and G11 carry λ prophage; KG110 was made lysogenic for P1.

Determinations of ampicillin degradation. The ampicillin (α-aminobenzylpenicillin, with D and L forms in a ratio of 6:4) was obtained from AB Astra, Södertälje, Sweden. It contained small amounts of decomposition products. Partly purified penicillinase from *B. cereus* (Neutrapen) was obtained from SchenLabs Pharmaceuticals, Inc., New York, N.Y. Strains tested for penicillinase or amidase activity were always grown in minimal medium. The reaction mixture contained per milliliter 10^{10} cells and 1.0 mg of ampicillin in 0.02 M phosphate buffer (*p*H 7.0). After 75 min of incubation at 37 C, a volume of 20 μliters was spotted on paper (Whatman no. 1 or 3MM) and developed, in cold room (4 C), with an ascending front for 4 to 6 hr. The solvent system was made up from 30 ml of *n*-butanol, 20 ml of ethyl alcohol, 20 ml of ether, and 30 ml of water. Ampicillin and the reaction products were localized by ninhydrin or by ultraviolet absorbancy.

For the spectrophotometric determination of penicillinase (Jansson, 1965), samples of the reaction mixture were filtered free of cells, diluted four times with the phosphate buffer, and kept in ice water until absorbancy was measured at 244 and 260 mμ. The difference between these readings was taken as a measure of the ampicillin concentration in the reaction mixture. The instrument used was a Zeiss QII spectrophotometer.

Media. The basal medium E (Vogel and Bonner, 1956) was used in all cases when a minimal medium was needed. It was always supplemented with vitamin B_1 (1 μg/ml) and with required amino acids (normally 25 μg/ml of the L isomer). The carbon source was usually 0.2% glucose. Plates were solidified with 1.5% agar (Difco). To score for mannitol-fermenting recombinants, 1% mannitol and 0.0012% bromothymol blue were added to the L agar of Luria and Burrous (1957). The *arg*+ recombinants were normally selected on plates containing 0.2% of Arginine Microbiological Assay Medium (H. M. Chemical Co., Santa Monica, Calif.). For Fig. 4 the *ampA2* (a.a.med.), *argF*+, and *metB*+ recombinants were selected on plates containing the minimal requirements supplemented with a mixture of nine additional amino acids. Nutrient agar (Difco) was used for scoring antibiotic-resistant cells, unless otherwise stated. Tryptone broth contained (per liter) 10 g of Tryptone (Difco) and 5 g of NaCl. Conjugation experiments were performed in medium made up from 20 ml of 0.5 M phosphate buffer (*p*H 6.4), 4 ml of aspartic acid (10 mg/ml, *p*H 7), 2 ml of 20% glucose, and Tryptone broth to a volume of 200

TABLE 2. *Isolation of some spontaneous ampicillin-resistant mutants**

Mutant strain	Parental strain	Selection on ampicillin	No. of cells per plate	Estimated frequency of mutants
		μg/ml		
G11b1	G11	2	5×10^7	10^{-5}
G11a1	G11	10	2×10^9	10^{-10}
G11d1	G11b1	50	4×10^9	10^{-11}
G11e1	G11a1	50	5×10^8	10^{-5}

* Mutants were selected on nutrient agar plates with ampicillin in the concentrations indicated. Pilot experiments were used to determine, for a given ampicillin concentration, the maximal number of cells which can be spread without giving confluent growth. All mutation experiments were started from single-cell colonies, and only one mutant was saved from each experiment. The resistance of these strains is shown in Fig. 1.

ml. Cultures for crosses were always grown in this medium.

Mating conditions. Overnight cultures of Hfr and F^- strains were grown at 37 C without shaking. They were diluted with fresh medium, incubated on a rotatory shaker at 37 C, and grown to a density of about 6×10^8 cells per milliliter. For conjugation, usually 0.5 ml of Hfr and 9.5 ml of F^- cells were mixed in a 500-ml flask; the flask was incubated at 37 C without shaking. Samples were withdrawn at different times, and conjugation was interrupted by violent agitation for 1 min with a Vortex Jr. Mixer. Dilutions were done in 0.5% NaCl containing 0.1% Difco nutrient broth (Dil). When *str-r* was used for counterselection, Dil was supplemented with streptomycin (100 μg/ml) (AB Kabi, Stockholm, Sweden). Samples spread on plates always had a volume of 0.1 ml.

Transduction experiments. In general, the transduction experiments were performed as described by Lennox (1955). The phage used was P1bt (Gross and Englesberg, 1959), obtained from R. Helling. For preparation of phage stocks, 3×10^6 plaque formers (grown on *E. coli* B) were mixed with 0.3 ml of late-log-phase bacteria and 3 ml of soft agar. The mixture was poured on L agar (Luria and Burrous, 1957) containing 0.01 M $CaCl_2$ and 0.1% glucose. After 10 hr, confluent lysis was obtained, and the plates were extracted with 5 ml of 0.5% NaCl for 10 min (Adams, 1959). The phage suspension was sterilized with chloroform. Phage stocks were always assayed on plates with L agar containing 0.01 M $CaCl_2$. The indicator was *Shigella dysenteriae* Sh15, obtained from G. Bertani. Stocks of phage P1bt were grown on G11a1 and G11e1 for experiments and on AB325(λL26) for controls. The titers obtained with G11 strains were about 10^{10} plaque formers per milliliter, with AB325(λL26) somewhat lower.

The gene-expression experiments were performed as follows. The medium used for acceptor bacteria was always the L broth of Luria and Burrous (1957) supplemented with 0.1% glucose. The fully grown acceptor bacteria were centrifuged and suspended in 0.5% NaCl containing 0.01 M $CaCl_2$, giving a final density of about 10^{10} cells per milliliter. Phage was added to give about one plaque former per bacterium, and the mixture was incubated at 37 C for 15 to 25 min. This period would allow time for phage adsorption and deoxyribonucleic acid injection without permitting any growth of the bacteria. The mixture was then diluted with 10 ml of 0.5% NaCl and centrifuged. The supernatant fluid was discarded; the bacteria were resuspended, and are hereafter referred to as "infected cells."

RESULTS

Determination of ampicillin resistance. Most penicillin-resistant bacterial strains seem to owe their resistance to the ability to produce enzymes which hydrolyze penicillin to products with negligible antibiotic activity. Since more cells produce more enzyme, estimates of penicillin resistance (as minimal inhibitory concentration) are usually strongly dependent on the size of the inoculum. We have previously tried to overcome this dependence by relatively rapid estimates of the penicillin concentration which gives lysis within one generation (the LIOG value described by Boman and Eriksson, 1963). More recently, we have obtained strains which destroy ampicillin too fast for the application of this method. Since the smallest and most well-defined inoculum will be a single colony former, in this study we have estimated the resistance as the ability to form colonies on nutrient agar plates with different concentrations of ampicillin. When a constant low number of cells (normally 100 to 300 colony formers) was spread on such plates, it was found for each strain that the number of colonies was constant up to a characteristic concentration of ampicillin and then decreased to zero within a relatively sharp interval (Demerec, 1945). Figure 1 gives the results from such determinations of ampicillin resistance for the F^- strain AB325, for the Hfr strain G11, and for four different spontaneous mutants of G11. AB325 did not grow on 1 μg/ml, whereas G11a1 grew on 10 μg/ml and G11e1 on 50 μg/ml. The differences between these strains were large enough to permit genetic experiments with clearly different selection conditions.

It was relatively easy to isolate ampicillin-resistant mutants in strains P4x and G11. In other strains, such as AB312, AB313, AB325, and AB673 (Taylor and Adelberg, 1960), we have been unable to isolate any one-step mutants which grow on plates with 10 μg of ampicillin per milliliter. The locus responsible for resistance to 10 μg/ml of ampcillin will be designated *ampA*. The mutant phenotype will be abbreviated amp^r_{10}. For the mutant resistant to 50 μg/ml of ampicillin, the phenotype will be designated amp^r_{50}.

Conjugation experiments with Hfr strains resistant to an ampicillin concentration of 10 μg/ml. The mutants previously described (Boman and Eriksson, 1963) were all derived from Hfr H, which transfers chromosome markers as follows: *(O)-thr-lac-his*. With these strains, no transfer of genetic determinants for ampicillin-resistance could be demonstrated. Preliminary experiments with strain P4xa2 showed that a mapping of *ampA* was possible, by use of the interrupted-conjugation technique of Wollman, Jacob, and Hayes (1956). The long time required for the entrance of *ampA* first led us to believe that the locus was close to *serA* (*ser/gly*). This was tested in a cross, P4xa2 $\times$ AB325 (Fig. 2),

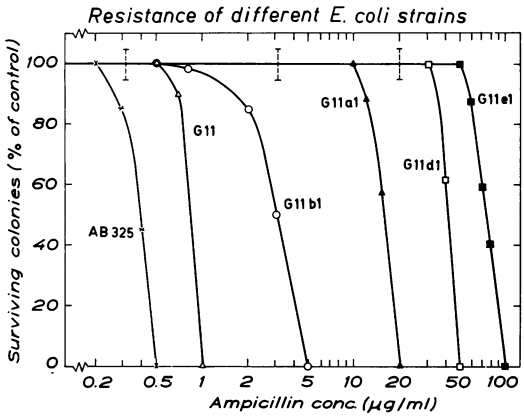

FIG. 1. *Ampicillin resistance of six strains of Escherichia coli K-12. Nutrient agar plates with increasing concentrations of ampicillin were seeded with 100 to 300 colony formers and incubated for about 18 hr at 37 C. The strains are described in Tables 1 and 2.*

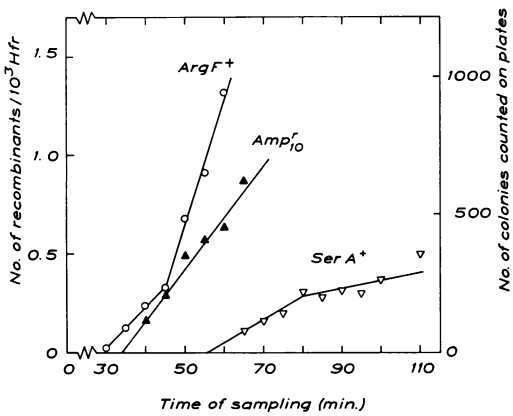

FIG. 2. *Kinetics of chromosome transfer by donor strain P4xa2 to recipient strain AB325.*

which showed *ampA2* to enter about 20 min before *serA*, but only 5 min after *argF*. An even closer linking between *ampA1* and *argF* was inferred from crossing G11a1 with AB325(λL26) (Fig. 3), with *mtl* as a second point of reference.

Figures 2 and 3 show that *ampA1* and *ampA2*, two independent mutations in different strains, mapped in the same region of the chromosome. In both of these experiments, the times between the reference markers were somewhat longer than the times given by Taylor and Thoman (1964) in their detailed study of the *E. coli* K-12 map. This may, however, be expected, since P4x and G11 inject *pro* and *lac* as their respective first genes, and since it is known that the rate of chromosomal transfer decreases after about one-

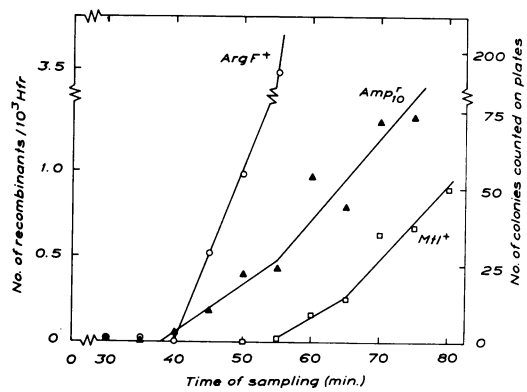

FIG. 3. *Kinetics of chromosomal transfer by donor strain G11a1 to recipient strain AB325(λL26).*

TABLE 3. *Segregation of ampA in serA⁺ str-r recombinants**

Crossover type	Unselected markers				No. of recombinants	Frequency of type
	mtl	*metB*	*argF*	*ampA*		
						%
F⁻	−	+	−	s	131	66.2
Hfr	+	−	+	r	8	
1	−	−	+	r	12	12.9
	+	+	−	s	15	
2	−	+	+	r	1	1.4
	+	−	−	s	2	
3	−	+	−	r	15	9.5
	+	−	+	s	5	
2 + 3	−	+	+	s	5	2.4
	+	−	−	r	0	
1 + 2	−	−	−	s	1	0.5
	+	+	+	r	0	
1 + 3	−	−	+	s	11	5.7
	+	+	−	r	1	
1 + 2 + 3	−	−	−	r	0	1.4
	+	+	+	s	3	

* In a cross P4xa2 × AB325 (λL26), 210 recombinants were selected after 90 min of mating. Colonies were purified once on the same type of plates as used for the selection. Unselected markers were tested by replica plating. Mannitol fermentation was tested in liquid cultures. Type 1 represents crossovers between *mtl* and *metB*; 2 between *metB* and *argF*; 3 between *argF* and *ampA*. The order of the loci are derived in part from the data shown in the table. Other orders were tried but did not fit the data. Abbreviations are the same as in Table 1.

third of the genome has entered (Jacob and Wollman, 1958).

We investigated the pattern of segregation of the *ampA* locus without the use of selection for ampicillin resistance. In a cross of P4xa2 ×

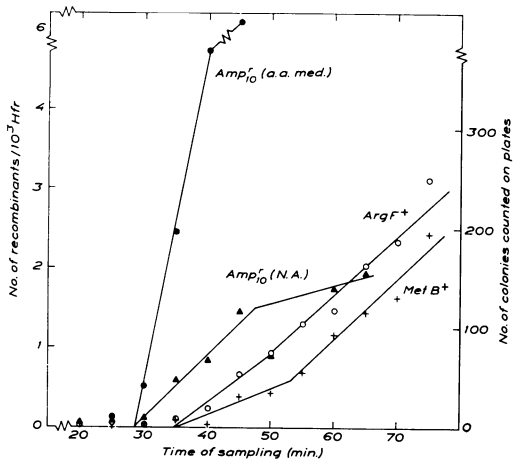

FIG. 4. *Kinetics of chromosomal transfer by donor strain P4xa21 to recipient strain KG110(P1). Ampicillin-resistant recombinants were selected on amino acid medium (a.a. med.)* (●) *and on nutrient agar (N.A.)* (▲), *both with an ampicillin concentration of 10 μg/ml.*

AB325(λL26), selection was made for *serA*$^+$ *str-r* recombinants. Of these cells, 210 were purified once and then tested with replica plating (Lederberg and Lederberg, 1952) for their unselected markers. The results (Table 3) show a rather uneven distribution of the two parental types. However, an excess of the F$^-$-like recombinants may be expected from our selection conditions, giving a crossover from *str-r* to *serA*$^+$ and a second crossover back to the F$^-$ chromosome. It is evident from Table 3 that *ampA* segregates as other unselected markers and that *metB* and *argF* are more closely linked than *argF* and *AmpA*. Assuming an even pairing, the distance between *argF* and *ampA* was estimated to be about 5 min.

It was pointed out to us that the *serA* marker would often decrease the general vitality of a strain (Lavallé, *personal communication*). To minimize the influence of general viability factors from other regions of the chromosome, crosses were performed with two strains in which *serA* and some other markers had been eliminated. In a cross between P4xa21 and KG110(P1), *ampA2* recombinants were selected on an amino acid medium as well as on nutrient agar, both supplemented with ampicillin and streptomycin. A considerably larger number of *ampA2 str-r* recombinants were obtained when an amino acid medium was used instead of nutrient agar (Fig. 4). Cells growing with ampicillin in an amino acid medium are lysed more slowly than in nutrient broth (Boman and Eriksson, 1963). If the production of ampicillin-destroying enzyme is independent of the medium, then a constant number of cells can be expected to hydrolyze more ampicillin per generation on an amino acid than on a rich medium. We previously found that penicillinase-producing cells can rescue nonresistant neighboring cells on plates. To exclude the possibility that F$^-$ cells had been mistaken to be recombinants, 232 *ampA2 str-r* colonies from amino acid medium were transferred to nutrient agar plates and tested by replica plating onto agar containing 10 μg/ml of ampicillin. All the colonies were found to be ampicillin-resistant.

In addition to the *argF* previously used as reference, we also scored for *metB*$^+$ *str-r* recombinants. Figure 4 shows a close linking between *argF* and *metB*, which is in good agreement with our finding of about 20% contransduction between these genes. Taylor and Thoman (1964) estimated the distance between *argF* and *metB* to be 1.5 min.

Time required for phenotypic expression of ampA. A lag period for the phenotypic expression of the *ampA* locus would give rise to a systematic error in the mapping in the above-described experiments. We therefore compared the expression of the *ampA* locus and the *argF* gene.

A cross was performed in the following way: strains P4xa2 and AB325(λL26) were mixed as during an ordinary conjugation. To kill the Hfr without interfering with the chromosomal transfer, streptomycin was added after 30 min to a final concentration of 100 μg/ml (Hayes, 1964). After 45 min, the conjugation was interrupted by violent agitation. A sample of the culture was diluted 50 times with medium containing streptomycin, and was incubated at 37 C. From this culture, samples of 0.1 ml were removed every 15 min, and were spread on selective media for scoring of recombinants and for viable counts. The results (Fig. 5) show that *ampA2* and *argF*$^+$ behaved in a similar way. For both of the genes, expression continued for at least 90 min; there may have been a short lag before the genes were expressed.

Transfer of ampicillin resistance by transduction. The transduction experiments were undertaken for three purposes: to determine whether *ampA* represented a chromosomal segment small enough to be transduced, to estimate the time required for phenotypic expression of *ampA*, and to score for possible cotransduction of *argF*$^+$ and *ampA*.

A culture of AB325(λL26) was treated with phage P1bt grown on G11a1. The infected cells were diluted with L broth (without added calcium) and incubated at 37 C; at various times, samples were withdrawn for scoring of transductants and for counts of viable cells. The

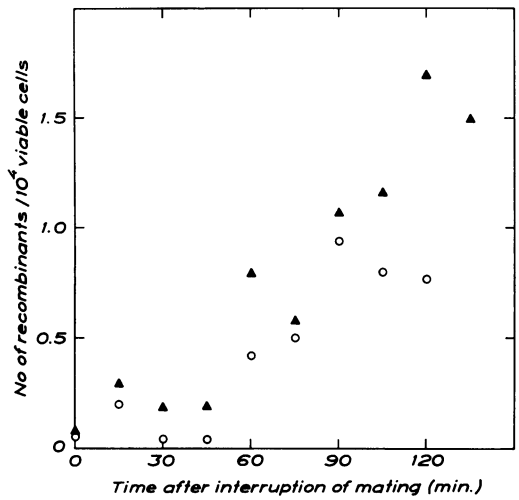

FIG. 5. *Comparison of the phenotypic expression of the ampA locus (▲) and the argF+ gene (○) after transfer of part of the chromosome from P4xa2 to AB325 (λL26).*

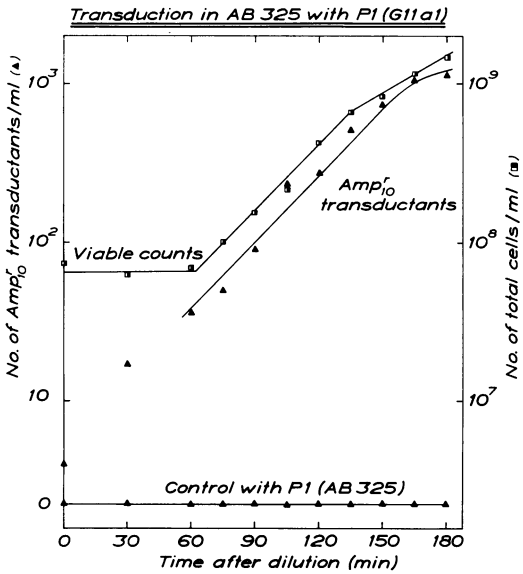

FIG. 6. *Growth and appearance of ampicillin-resistant transductants from a culture of AB325 (λL26) infected with P1bt grown on G11a1. The numbers of transductants at 0 and 30 min are too low to permit conclusions about the beginning of the curve. The control was infected with P1bt grown on AB325(λL26).*

frequency of *ampA1* transductants was about 5×10^{-7} per recipient bacterium (Fig. 6). The control with homologous phage gave no resistant cells; this shows that our noise level of mutations is lower than found by Banič (1959). The experiment demonstrates that *ampA1* can be transduced and that the locus therefore represents less than 2% of the entire chromosome (Hayes, 1964).

There was a 60-min lag period before the recipient bacteria started growing (Fig. 6). This lag varied from one experiment to the other, but in all cases the *ampA1* transductants started to appear just before or at the onset of growth. This indicates that the *ampA* locus is expressed without a significant lag period. In a comparable experiment with the expression of *str-r*, a lag of about three generations was obtained, in agreement with previous findings (*see* Hayes, 1964).

Attempts to demonstrate a cotransduction of *ampA* have so far been unsuccessful. Phage P1 grown on G11a1 was used for transducing the *argF*+ gene into AB325; among more than 1,000 *argF*+ transductants, none was found to be resistant to ampicillin.

Experiments with a strain resistant to an ampicillin concentration of 50 μg/ml. Several attempts were made to transfer genetic material from the highly ampicillin-resistant strain G11e1 to the sensitive strain AB325(λL26). When G11e1 was used as donor strain in transduction experiments, only moderately resistant colonies of the amp^r_{10} phenotype were obtained. No colonies were found on plates with 50 μg of ampicillin per ml. This is in agreement with the results of Banič (1959) and the interpretation that step-wise increasing penicillin resistance is controlled by a polygenic system.

In an interrupted-conjugation experiment with G11e1 and AB325(λL26), selection was made for recombinants of both the amp^r_{10} and the amp^r_{50} phenotypes. The *argF*+ gene was used as reference. Figure 7 shows that only amp^r_{10} recombinants were obtained. As in the experiments previously described, this locus was linked to the *argF* gene. Selection after 2 hr in a cross between G11e1 (amp^r_{50}) and KG3 (which carried *ampA2*) failed to give any amp^r_{50} recombinants. Thus, assuming that the difference between G11a1 and G11e1 is due to an additional mutation in a locus different from *ampA*, it seems unlikely that this second locus is to be found in the chromosomal region *pro-ilva-serA*.

Estimates of the penicillinase activity of cell suspensions. Paper chromatography was used to study the qualitative degradation of ampicillin by whole-cell suspensions. Figure 8 shows the ninhydrin spots obtained on two chromatograms. The references with only ampicillin (no. 5 and 14) showed a main bluish spot with R_F about 0.5, and a minor brownish one with R_F about 0.3. Ampicillin incubated with partly purified penicillinase from *B. cereus* (no. 1 and 15) gave

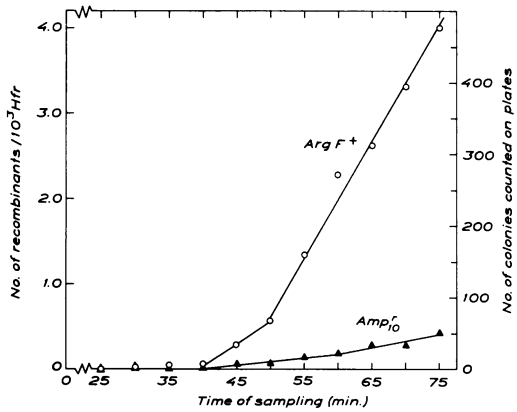

FIG. 7. *Kinetics of chromosomal transfer by donor strain G11e1 (phenotype amp$^r_{50}$) to recipient strain AB325(λL26). No colonies were obtained on nutrient agar plates with 50 μg of ampicillin per ml.*

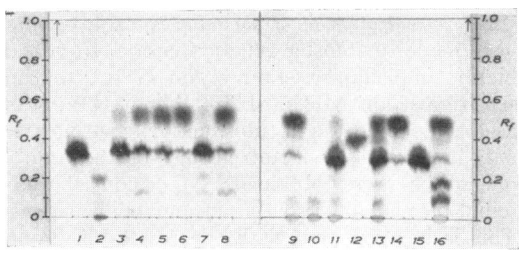

FIG. 8. *Paper chromatograms of ampicillin incubated for 75 min at 37 C with suspensions of whole cells. Left paper, Whatman no. 3MM; right, Whatman no. 1. Spot numbers: 1, ampicillin + penicillinase from Bacillus cereus; 2, control with P4xa2 cells without ampicillin; 3, P4xa2 + ampicillin; 4, AB325(λL26) + ampicillin; 5, ampicillin reference; 6, P4x + ampicillin; 7, KG3 + ampicillin; 8, KG110(P1) + ampicillin; 9, G11 + ampicillin; 10, control with G11a1 cells without ampicillin; 11, G11a1 + ampicillin; 12, 6-aminopenicillanic acid reference; 13, G11e1 + ampicillin; 14, ampicillin reference; 15, ampicillin + penicillinase from B. cereus; 16, AB325(λL26) + ampicillin.*

only the latter spot. An increase in this spot was therefore taken as evidence for penicillinase activity. The reference with 6-aminopenicillanic acid (no. 12) gave a faintly brownish spot, with R_F about 0.4. Such a spot produced from ampicillin is consistent with the presence of amidase activity. Considering these references, Figure 8 indicates that P4x, G11, and AB325(λL26) were lacking penicillinase (see no. 6, 9, 4, and 16), whereas the one-step mutants P4xa2 and G11a1 (no. 3 and 11) produced penicillinase. This enzyme was also present in strain G11e1 (no.

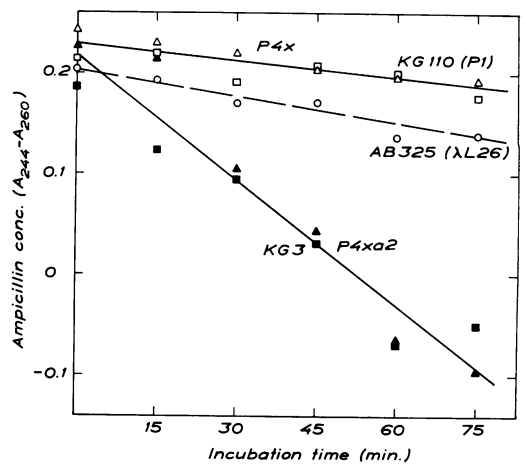

FIG. 9. *Penicillinase activity of suspensions of whole cells grown in minimal medium. Strains P4xa2 (▲) and KG3 (■) carry the ampA locus, whereas P4x (△), AB325(λL26) (○), and KG110 (P1) (□) are ampicillin-sensitive. P4xa2 is a one-step mutant of P4x. KG3 and KG110(P1) are recombinants from a cross of P4xa2 × AB325(λL26).*

13), which in addition showed evidence for amidase activity.

A direct spectrophotometric assay of penicillinase was recently described (Jansson, 1965). The method depends on a decrease in the penicillin absorbancy at 244 mμ, produced by the hydrolysis of the β-lactam ring. To adapt the method to measurements on suspensions of whole cells, it was necessary to subtract the absorbancy of the products excreted by the cells during the determination. Figure 9 shows the time curves obtained for the hydrolysis of ampicillin by suspensions of the sensitive strains P4x and AB325(λL26), the resistant mutant P4xa2, and two recombinants from a cross of P4xa2 and AB325(λL26). For all five strains, there was thus a correlation between the presence or absence of penicillinase activity and the degree of resistance on plates. The 75-min samples from the incubation mixture (Fig. 9) were used for spots no. 3–8 in Fig. 8.

DISCUSSION

Expression of the ampA locus and its mapping on the chromosome. Riley et al. (1960) showed that the structural gene for the enzyme β-galactosidase was expressed without any significant lag period after transfer in a conjugation experiment. The situation would be expected to be the same in the case of the *argF* gene, and related in the case of the *ampA* locus (assuming this to be a structural gene). However, during selection the

absence of β-galactosidase or an arginine enzyme prevents growth, whereas the absence of an ampicillin-destroying enzyme may be lethal. For this reason, and since nothing yet is known about a hypothetical induction of the *ampA* locus product(s), care must be exercised in the interpretation of the analogous behavior, demonstrated in Fig. 5, of the *argF*$^+$ gene and the *ampA* locus.

Effects of medium on the number of recombinants have been reported by Haan and Gross (1962) and by Schneider and Falkow (1964). Figure 4 showed that the medium had a significant influence on the slope of the curve for *ampA* recombinants. However, the extrapolated time-of-entry value was unaffected by the medium, which therefore can hardly give a systematic error in the mapping.

Another factor to consider in the judgment of the accuracy of the mapping is the anomalous recombinant curves obtained in almost all interrupted-conjugation experiments. Taylor and Thoman (1964) and Schneider and Falkow (1964) discussed such "broken slopes," and our data do not exclude any of their explanations. Most of our experiments indicate that *ampA* is located to the right of the *argF* gene used as reference. If the maximal error were 5 min, it should be possible to place the *ampA* locus between the *argF* and *pyrB* (Fig. 10) when referring to the map of Taylor and Thoman (1964). This location of *ampA* is supported by the results in Table 3, in which any influence of the phenotypic expression and "broken slopes" is eliminated. Weaker evidence for the location may be the negative results obtained by mating experiments with an ampicillin-resistant mutant derived from Hfr H, as well as the failure to obtain cotransduction between *ampA* and *argF*. (The authors would be grateful for suggestions, addressed to H. G. B., about strains with published or unpublished markers suitable for a cotransduction mapping of the *ampA* locus.)

On the possible biochemical mechanism underlying a stepwise increasing penicillin resistance. The following biochemical phenomena (*see* review by Moyed, 1964) can be expected to result in a stepwise increased resistance to ampicillin: (i) production of the enzyme penicillinase, in which case one can expect the involvement of a structural gene for the enzyme and one or more genes concerned with the regulation of the enzyme synthesis (Echols et al., 1961); (ii) production of an enzyme which can cleave off the side chain of ampicillin (English et al., 1960), analogous to the production of penicillinase, considering the number of genes involved; (iii) overproduction of a natural cell-wall metabolite, with which

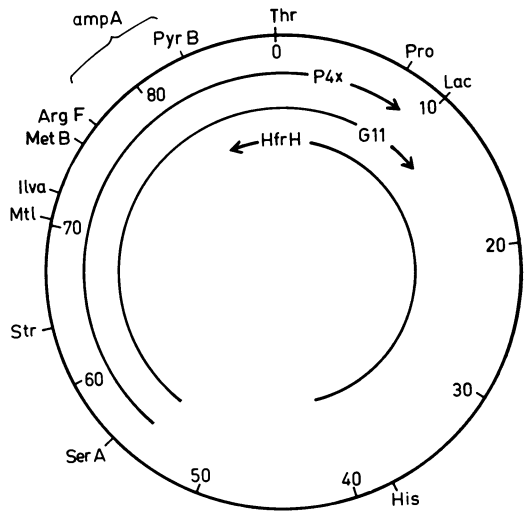

FIG. 10. *Genetic map of Escherichia coli, with a diagrammatic representation of the chromosomal segments used for the mapping of the ampA locus. Time indications and markers according to Taylor and Thoman (1964).*

ampicillin is assumed to compete (the A-X suggested by Boman and Eriksson, 1963), this case perhaps including the participation of several structural and regulatory genes for a pathway of cell-wall biosynthesis; (iv) the involvement of both a chromosomal system and an episomal system for drug resistance (Watanabe 1963); and (v) any combination of these.

Our present results indicate that the parent strains G11 and P4x had a negligible ampicillin-destroying activity, whereas the two independent one-step mutants G11a1 and P4xa2 had penicillinase activity, presumably controlled by the *ampA* locus. For the two-step mutant G11e1, the situation is more complex. The evidence available indicates that this strain has both penicillinase (it carries the *ampA* locus) and amidase activity. In addition, our strains were found to excrete compounds of unknown nature. If ampicillin acts as an antimetabolite of cell-wall biosynthesis, it can be expected to undergo several conversions and to interfere with several enzymes. It could then be possible that the nonmutated enzymes which correspond to penicillinase and amidase are normal metabolic enzymes of cell-wall biosynthesis. These assumptions would account for the occurrence of penicillinase after a one-step mutation. Two likely consequences of the hypothesis would be that the natural substrates of the nonmutated enzymes are present among the excreted compounds and show protective activity against lysis caused by penicillins.

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