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Resistance of *Escherichia coli* to penicillins

IV. Genetic study of mutants resistant to D,L-ampicillin concentrations of 100 μg/ml.

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1. INTRODUCTION

The sensitivity of *Escherichia coli* to D,L-ampicillin differs somewhat from strain to strain. On plates with rich medium, the average tolerated concentrations lie between 0·5 and 2 μg/ml. The development of resistance to ampicillin is a stepwise process which involves more than one locus. First step mutants can grow well on D,L-ampicillin concentrations of 10–25 μg/ml. This resistance is the result of a mutation in the *ampA* gene, located near 80 min* (Eriksson-Grennberg, Boman, Jansson & Thorén, 1965). Further mapping has localized *ampA* close to 82 min and provided a system for cotransduction between *ampA*, *purA* and *fdp* (Eriksson-Grennberg, 1968). *AmpA* strains produce a penicillinase for which a preliminary purification has been worked out (Lindström & Boman, 1968).

A second class of mutants has been studied which in addition to *ampA* contains a mutation, *ampB*, at a second site which is located at least 20 min from *ampA* (Eriksson-Grennberg *et al.* 1965). The resistance of these mutants is 2–3 times that of *ampA* strains. Utilizing the cotransduction between $ampA^+$ and $purA^+$, a strain with only *ampB* was produced (Boman, Eriksson-Grennberg, Földes & Lindström, 1967). It was demonstrated that *ampB* can enhance the resistance of both *ampA* and its wild type allele as well as of R-factors (Nordström, Eriksson-Grennberg & Boman, 1968).

This paper deals with a third class of *ampA*-containing mutants (designated *ampAB*) which are resistant to D,L-ampicillin concentrations of 100 μg/ml. Such an F^- strain crossed to sensitive Hfr strains injecting the chromosome counter clock-wise gave five classes of resistant $proB^+$ recombinants. Hfr strains with the reversed order of injection gave only two types of resistant trp^+ recombinants. In transduction experiments with selection for *ampA* some of the cells received the full resistance of the *ampAB* donor. Ampicillin resistant strains showed some mating abnormalities which indicate that the cell surface was changed. Some of the strains also showed a poor ability to propagate an RNA phage.

* Map positions in min refer to the time scale of Taylor & Dunham Trotter (1967).

2. MATERIALS AND METHODS

(i) *Organisms*

All strains used were *Escherichia coli* K12, and the main derivatives are listed in Table 1. Strains D11, D21, and D31 are derived from strain RC711 of Meynell & Datta (1966) in the following way. In D1 (= RC711) the *ampA1* allele was introduced by transduction. The transductant obtained, D2, was grown for about three generations in the presence of ethyl methane sulfonate (EMS) at a concentration of 100 μl per 10 ml of LB medium. After removing EMS by centrifugation a clone was selected which could grow on LA plates with ampicillin concentrations of 100 μg/ml (strain D3). As final steps EMS treatment of D1, D2 and D3 produced the streptomycin resistant derivatives D11, D21, and D31. Strain D31 could grow in minimal medium when proline was replaced by glutamic γ-semialdehyde (kindly provided by Dr A. Baich). This observation rules out that D31 contains *proC* and is consistant with the information that RC711 contains *proB* (W. Hayes, personal communication). Strain G11h5 was obtained as a spontaneous mutant of G11a1 selected on a plate with a D,L-ampicillin concentration of 100 μg/ml. AB311a1 is a transductant of AB311 obtained with G11a1 as donor. AB311t6 was a transductant of AB311 using D3 as donor. AB311a1 was sensitive to superinfection with P1, while AB311t6 was immune.

The P1 used for transduction was P1bt obtained from Dr R. Helling. Other phages used were normal wild types, propagated on strain AB311.

(ii) *Media*

All media are described in the preceding papers by Eriksson-Grennberg (1968) and Nordström *et al.* (1968). LA plates normally contained 0·0025 M-$CaCl_2$.

(iii) *Standard mating procedure*

Strains to be mated were first grown at 37 °C in LB to an optical density of 100 Klett units (about 4×10^8 cells/ml). Of these cultures 10 ml F^- cells were mixed with 1 ml Hfr cells in a prewarmed 300 or 500 ml flask and rotated very slowly at 37°. Samples of 0·1 ml were withdrawn at intervals and added to 0·2 ml T6 suspension (titre $2–4 \times 10^{10}$ plaque-forming units/ml). After 3 min at room temperature the sample was diluted with 5 or 10 ml 0·9% NaCl, blended with a Vortex Super-mixer for 1 min and 0·1 ml was spread on plates. When T6 was not used, the sample was immediate diluted and blended.

Recombinants were always purified once on the selective media used for isolation. If saved and tested by single-cell-colony formation they were purified a second time.

(iv) *Transduction*

Stocks of P1 were prepared as described by Eriksson-Grennberg (1968). After phage adsorption and DNA injection during 30 min at 37° C, the bacteria were washed once and spread on LA plates containing ampicillin but not $CaCl_2$. Immunity to P1 was tested as described by Nordström *et al.* (1968).

Table 1. *Strains of* Escherichia coli *K12 and their relevant characters**

Strain	Reference	Sex	λ-Prophage	Response to T6	Response to str	Ampicillin phenotype†	Ampicillin genotype: Site 1	Ampicillin genotype: Site 2	Other relevant markers
D11	This paper	F⁻	+	r	r	amp-s	+	+	*proB, trp, his*
D21		F⁻	+	r	r	amp-10	*ampA1*	+	*proB, trp, his*
D31		F⁻	+	r	r	amp-50	−	*amp(A1B4)1*	*proB, trp, his*
AB311‡	Taylor & Adelberg (1960)	Hfr	−	s	r	amp-s	+	+	*thr, leu*
AB311a1	This paper	Hfr	−	s	r	amp-10	*ampA1*	+	*thr, leu*
AB311t6		Hfr	−	s	r	amp-50	*amp(A1B4)1*/++ heterogenote		*thr, leu*
G11‡	Stent & Brenner (1961)	Hfr	+	s	s	amp-s	+	+	*metB, ilv*
G11a1	Eriksson-Grennberg	Hfr	+	s	s	amp-10	*ampA1*	+	*metB, ilv*
G11e1	*et al.* (1965)	Hfr	+	s	s	amp-20	*ampA1*	*ampB1*	*metB, ilv*
G11h5	This paper	Hfr	+	s	s	amp-50	Unknown		*metB, ilv*
Hfr H‡	Hayes (1964)	Hfr	−	.	s	amp-s	+	+	
Hfr 6‡	B. Low (unpublished)	Hfr	−	.	s	amp-s	+	+	*met*
KL99‡		Hfr	−	.	s	amp-s	+	+	

* Abbreviations: amp, ampillicin; gal, galactose; his, histidine; ilv, isoleucine-valine; lac, lactose; leu, leucine; met, methionine; pro, proline; pur, purine; str, streptomycin; thr, threonine; trp, tryptophan; tsx, phage T6; s = sensitivity; r = resistance; The capital letters after some of the symbols refer to the genetic map of Taylor & Dunham Tropper (1967). Thiamine was always present in our growth media and all G11 strains are thiamine requiring.

† The phenotype is designed amp-n, where n denotes the relative increase in resistance. Amp-s varies somewhat from strain to strain.

‡ G11 is a derivative of Hfr Cavalli which injects 0-*purE-proB-leu*. The other Hfr strains transfer genes as follows: AB311 0-*his-trp-proB*; Hfr H 0-*thr-proB-trp*; Hfr 6 0-*purE-trp-his*; KL99 0-*pyrD-trp-his*. The last strains were kindly obtained from Dr B. Low.

(v) *Ampicillin and determinations of ampicillin resistance*

The ampicillin used in this study was kindly supplied by Astra, Södertälje, Sweden and contained D- and L-epimers in the ratio 2:3. In previous studies (Eriksson-Grennberg *et al.* 1965) another preparation of ampicillin was used in which the ratio between D- and L-epimers was 3:2. Since the L-epimer is less active in lysing *E. coli* (Boman & Eriksson, 1963) and is also less stable chemically (Sjöberg & Östergren, unpublished) the resistance figures obtained in this study are not strictly comparable to those previously obtained. Resistance was tested by replication or by single-cell-colony formation on LA plates with different concentrations of ampicillin (for a recent comparison of the D- and D,L-epimers of ampicillin, see Nordström *et al.* 1968). Since replication is less accurate than single-cell-colony formation as a method of measuring resistance (r), the results of replication are given in the form $10 < r < 50$, which means that growth was obtained on 10 but not on 50 μg/ml. Phenotypes are given as amp-s for wild type and Amp-n for mutants, where n gives the relative increase in the resistance of the mutant. Phenotypes were always tested by single-cell-colony formation.

3. RESULTS

(i) *Crosses with Hfr strains injecting the chromosome counter clock-wise*

The resistant strain D31 was first crossed with two ampicillin-sensitive Hfr strains, B7 and B8 (Broda, 1967). During the work both B7 and B8 reverted to the F^+ state. Despite this complication it could tentatively be concluded that the genes responsible for higher resistance were located between *proB* and *trp*.

To confirm the location, D31 was mated to Hfr AB311 which injects its chromosome 0-*his-trp-proB*. This cross and all others were performed as interrupted mating experiments. The very early times served as controls for remating, while time of entrance of the first marker, the order of the markers, and the recombination frequency provided controls of the properties of the Hfr strains used. In this experiment three types of recombinants were selected, namely *his*$^+$, *trp*$^+$ and *proB*$^+$, with counterselection for *thr*$^+$ and *leu*$^+$, and using T6 killing of the Hfr.* After purification the recombinants were tested by replication for their ampicillin resistance. The results of cross 1 in Table 2 show that all the *his*$^+$ recombinants had retained the same resistance as D31. However, among the *trp*$^+$ recombinants 12% had segregated into lower levels of resistance and for the *pro*$^+$ recombinants this figure was 32%. The experiment was therefore consistent without preliminary conclusions that some of the genes for ampicillin resistance are located in the region *trp-proB*. Since D31 contains the *ampA1* allele which in one-step mutants maps near 82 min (Eriksson-Grennberg, 1968), it was unexpected that 3% of the *trp*$^+$ and 14% of the *proB*$^+$ recombinants were ampicillin-sensitive.

In an attempt to rule out that the sensitive strains were produced as a result

* All experiments described here were planned under the assumption that in D31 the gene order from 0 to 12 min was *thr-lac-tsx-pro* (Taylor & Thoman, 1964).

Table 2. *Analysis of recombinants from crosses with derivatives of AB311*

Cross No.	Hfr × F⁻ phenotypes	Selection for	No. of tested recombinants	Percentage recombinants with resistance (r)			
				0 < r < 10	10 < r < 50	50 < r < 100	r ⩾ 100
1	—	*his*⁺	164	0	0	0	100
	AB311 × D31	*trp*⁺	158	3	6	3	88
	amp-s × amp-50	*proB*⁺	107	14	12	6	68
2	—	*his*⁺	100	0	0	0	100
	AB311a1 × D31	*trp*⁺	149	2	0	0	98
	amp-10 × amp-50	*proB*⁺	142	10	0	4	86

Thr⁺*leu*⁺ was used as counter-selection in both crosses. T6 was used for killing of the Hfr. The recombinants selected for further testing are listed in Table 5 and can be traced by the information given below this Table. Resistance (r) was tested by replication and the numbers refer to the concentration of D,L-ampicillin (2:3) on which growth was respectively obtained and not obtained.

Table 3. *Crosses with derivatives of Hfr Cavalli and selection for* proB⁺/str-r

Cross No.	Cross Hfr × F⁻	Phenotypes Hfr × F⁻	T6 killing	No. of recomb. per 15 min. per 0·1 ml	No. of tested recombinants	Percentage recombinants with resistance (r)			
						0 < r < 10	10 < r < 50	50 < r < 100	r ⩾ 100
3	G11 × D31	amp-s × amp-50	−	3×10^4	170	1·2	0	0·6	98·2
4	G11 × D31	amp-s × amp-50	+	8×10^4	100	1	0	2	97
5	G11a1 × D31	amp-10 × amp-50	+	7×10^4	148	0	0	13·5	86·5
6	G11e1 × D21	amp-20 × amp-10	+	4×10^4	146	0	100	0	0
7	G11h5 × D11	amp-50 × amp-s	−	3×10^4	100	99	1	0	0
8	G11h5 × D21	amp-50 × amp-50	+	3×10^3	150	0·7	99·3	0	0
9	G11h5 × D31	amp-50 × amp-50	−	3×10^4	205	0	0	9	91

The phenotypes with respect to ampicillin resistance give the relative increase in resistance provided by the respective genes (see Nordström *et al.* 1968). The two sensitive recombinants obtained in cross 3 (strains EM61 and EM62) were obtained after 15 min mating time. The sensitive recombinant produced in cross 8 is strain EM57. No differences were found in segregation pattern between recombinants selected at different times. For suggested genotypes, see Table 5. The number of recombinants per 15 min per 0·1 ml was obtained by a straight line interpolation of the number of recombinants versus time of sampling.

of recombination in the *ampA* region at 82 min the allele *ampA1* was transduced into AB311 giving strain AB311a1. When this strain was crossed to D31 (cross 2 in Table 2) 10% of the *proB*$^+$ recombinants were found to have a lower level of resistance than the donor strain AB311a1. To score for the presence of *ampB* in the sensitive recombinants resistance tests were performed with and without the R-factor, R1, as described by Nordström *et al.* (1968). It was found that eight of the sensitive recombinants carried the *ampB* allele while nine were indistinguishable from the wild type. The results of crosses 1 and 2 are difficult to explain assuming that *ampA* in D31 is in its original position near 82 min. However, the results are explainable by the hypothesis that D31 contains *ampB* and a chromosomal aberration (designated *ampAB*) by which *ampA* was moved to the *trp-pro* region.

Hfr Cavalli transfers chromosomal genes in the following order: 0-*purE-proB-leu*. G11 and derivatives were therefore used to investigate whether any of the genes responsible for ampicillin resistance were found in the *pro* region of the chromosome. Table 3 gives the result of crosses with seven different combinations of sensitive and resistant strains. In all experiments *proB*$^+$/*str-r* recombinants were selected and after purification tested for resistance. The main result of these experiments was that when the resistance genes were present in the recipient (crosses 3–5), most recombinants were of the recipient type. If the resistance was present in the donor (crosses 6–8), almost all of the recombinants had the sensitivity of the recipient. R-factor tests showed that the two sensitive recombinants produced in cross 3 (EM61 and EM62) both contained *ampB* while the sensitive recombinant obtained in cross 8 (EM 57) was of the wild type. In cross 9 two Amp-50 strains were crossed to each other. When recombinants selected for Amp-r $\geqslant$ 100 were tested for resistance by single-cell-colony formation several were found to be more resistant than the parents and to grow well on plates with 150 μg of ampicillin/ml. However, the resistance of these recombinants was unstable and lost after a few experiments.

It is essential for the interpretation of the crosses to investigate the number of classes of recombinants with respect to the resistance. For this reason the R-factor R1 was introduced into all recombinants and parent strains which were believed to differ from one another. Resistance with and without the R-factor was then estimated using the ability to form single-cell colonies on agar plates with different amounts of ampicillin. The results given in Table 4 indicate that five classes of cells were found. The table also includes the suggested genotypes which will be further treated in the discussion.

(ii) *Crosses with Hfr strains injecting the chromosome clock-wise*

We have performed crosses with the *ampAB* strain D31 and three different Hfr strains all sensitive to ampicillin and injecting the chromosome clock-wise, which is the reverse order to that of AB311 and Hfr Cavalli. Hfr Hayes (Hfr H) injects *thr* as a rather early marker and transferred *proB* after 15–17 min and *trp* after 40 min (crosses 10 and 11 in Table 5). Hfr 6 injects *purE* as an early marker and

transferred *trp* after 30–35 min (cross 12). Strain KL99 injects *pyrD* as an early marker and transferred *trp* after 14–15 min (cross 13). With the two first Hfr strains the transfer of *trp* was at least 10 min later than expected from the map of Taylor & Dunham Trotter (1967). The results in Table 5 show the resistance of the recombinants obtained. Only HfrH transfers the *proB* gene and none of the *proB*$^+$ recombinants showed any segregation into lower levels of resistance. All three strains transferred *trp* but only HfrH and Hfr 6 gave segregation into lower levels of resistance. The fact that no segregation was obtained with KL99 would indicate that the wild type genes corresponding to the resistance genes were not transferred with this Hfr strain.

Table 4. *Classification of parental and recombinant strains with the aid of R-factor*

Group	Strains	Resistance to D,L-ampicillin (μg/ml) without R1	Resistance to D,L-ampicillin (μg/ml) with R1	Suggested genotype at Site 1	Suggested genotype at Site 2
I	G11; AB311; KG64(1); KG68(2); EM57(8)	0–4	100–200	+	+
II	EM61(3); EM62(3); KG41(1); KG45(1); KG71(2); KG93(2)	4–10	300–400	+	*ampB*
III	G11a1; AB311a1; D21; EM59(8); EM77(8); KG34(1)	10–50	100–200	*ampA*	+
IV	EM55(2); EM82(5); KG38(1)	50–100	200–300	*ampA*	*ampB*
V	D31	100–200	300–400	Deletion?	*ampAB*

For the strains which are recombinants in crosses described in this paper, the cross number is given within brackets immediately following the denotation of the strains. For most recombinants the recipient was D31, only recombinants from cross 8 are derived from D21. The following recombinants were selected as *proB*$^+$: KG68(2), EM57(8), EM61(3), EM62(3), KG45(1), KG71(2), EM59(8), EM77(3), KG34(1), EM55(5), EM82(5), KG38(1). The remaining recombinants were isolated as *trp*$^+$.

We have in all *ampAB* strains frequently observed segregation of clones which at room temperature give a heavy formation of capsule-like material. The difference in segregation of $50 < r < 100$ recombinants between crosses 10 and 11 (Table 4) was due to the fact that 46 of the recombinants picked in cross 11 were producing capsule. All but 1 of these had the full resistance of the recipient. Reeve (1968) has observed that some chloramphenicol-resistant mutants are mucoid. The region *proB-trp* is known to contain two regulatory genes involved in capsule formation (Markovitz, Lieberman & Rosenbaum, 1967). Mutations to phage resistance, which often are accompanied with capsule formation, have been shown by Curtiss (1965) to be chromosomal aberrations.

(iii) *Mating abnormalities produced by* ampAB

During an analysis of the kinetics of the crosses given in Table 3 it was observed that the entering time for *proB*$^+$ varied in a way which seemed to depend on the

Table 5. *Analysis of recombinants from crosses with Hfr Hayes, Hfr 6 and KL99*

Cross No.	Hfr × F⁻ phenotypes	Selection for	No. of tested recombinants	Percentage recombinants with resistance (r)			
				0 < r < 10	10 < r < 50	50 < r < 100	r ⩾ 100
10	Hfr H × D31	*proB*⁺	100	0	0	0	100
	amp-s × amp-50	*trp*⁺	100	0	0	6	94
11	Hfr H × D31	*proB*⁺	149	0	0	0	100
	amp-s × amp-50	*trp*⁺	100	0	0	55	45
12	Hfr 6 × D31 amp-s × amp-50	*trp*⁺	100	0	0	58	42
13	KL99 × D31 amp-s × amp-50	*trp*⁺	96	0	0	0	100

Str-resistance was used as counter-selection in all crosses. No phage was used for Hfr killing.
Ampicillin resistance (r) was tested by replication.

ampicillin resistance of the strains. Two alternative explanations of this effect were considered: (1) Mutations giving increased penicillin resistance also produce changes in the surface layers of the bacteria which in turn give rise to pairing difficulties during mating. (2) Mutations giving increased penicillin resistance are the result of chromosomal alternations, which affect the *pro* region of the chromosome and produce recombination difficulties.

In order to test the first hypothesis a series of crosses was performed using the mating technique of de Haan & Gross (1962). In these experiments pairing was allowed to occur during 5 min. The culture was then gently diluted 100-fold with prewarmed medium to prevent further pair formation. *ProB*$^+$ recombinants were selected at different times using streptomycin as counter selection. The first experiment was designed to test whether the presence of *ampAB* in the F$^-$ strain had any effect on mating. The same culture of the sensitive donor G11 was crossed to the *ampAB* strain D31 and, as a control, to its sensitive parent strain D11. Figure 1 shows that the entering time for *proB*$^+$ was the same in both crosses. The number of recombinants with D31 was about one-third of that obtained in the control. From these experiments it can be concluded that the presence of *ampAB* in the recipient reduces the number of recombinants, probably by decreasing the number of pairs which were held together strongly enough to resist the dilution (de Haan & Gross, 1962).

In the next experiment we investigated whether the presence of the *ampA* gene in the donor could produce additional pairing difficulties. Cultures of G11a1 and D31 were mixed as in a normal cross (see Materials and Methods). From the mixed culture, 0·5 ml was immediately transferred to a prewarmed flask and after 5 min of incubation it was carefully diluted with 50 ml of prewarmed medium. From the undiluted culture, samples were removed at different times, treated with T6, diluted 100-fold, and plated. From the diluted mating mixture, samples were removed for similar treatment with T6, but plated without further dilution. Figure 2 shows that the mating technique of de Haan and Gross and the undiluted culture gave the same extrapolated time for the entrance of *proB*$^+$. This time was 20 min compared to the 15 min recorded in Fig. 1, which indicates that *ampA* delays the entrance of *proB*$^+$. With the de Haan and Gross method the same number of recombinants was obtained in the cross G11 × D31 (Fig. 1) and in the cross G11a1 × D31 (Fig. 2). However, this number was consistently 4–10 times lower than the number of recombinants obtained by our usual mating procedure regardless of whether G11 or G11a1 was used as donor.

When de Haan and Gross' mating method was used with the *ampAB* donor strain G11h5, very few recombinants were obtained. To compare pair formation and chromosomal transfer with G11h5 and G11 we used the normal mating technique with D31 as recipient. In both experiments streptomycin resistance was used as counter selection with and without selection for *metB*$^+$ as well. This gene is located at 76 min near the normal position of *ampA*. Figure 3 shows that with G11h5 the transfer of *proB*$^+$ is considerably delayed when compared to G11. This delay is consistent with the fact that only very few recombinants were

obtained by the de Haan and Gross method. The experiment indicates that the presence of *ampAB* in both donor and recipient gives rise to significant pairing difficulties. The additional selection for methionine did not alter the kinetics or the distribution of any of the unselected markers tested for among the recombinants.

During the course of the work with derivatives of G11 it was several times observed that the kinetic curves showed humps of the type illustrated in Fig. 4. The interpretation was made that the donor culture had formed an episome carrying both *proB*$^+$ and *ampA*. For this reason several clones of G11a1 were tested but only clones producing normal kinetics could be recovered.

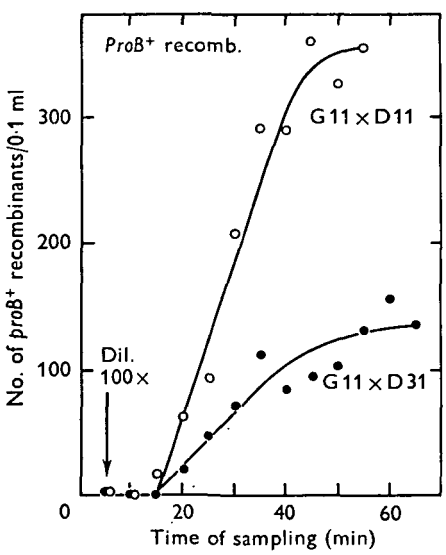

Fig. 1

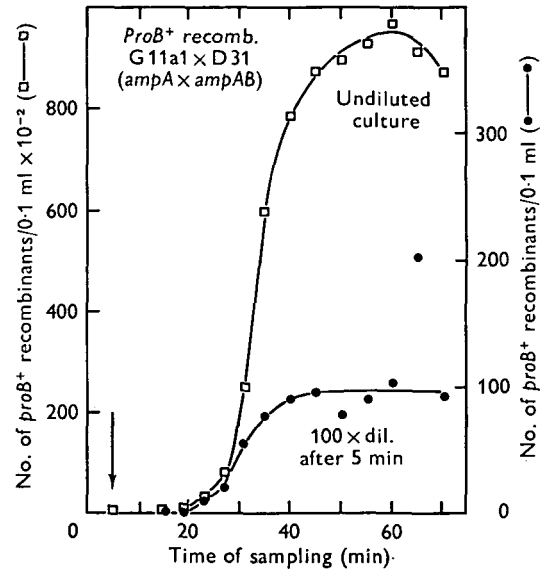

Fig. 2

Fig. 1. Kinetics for the entrance of *proB*$^+$ in the presence and absence of *ampAB* in the recipient. Both crosses were performed simultaneously and the same culture of the donor G11 was used. After 5 min both cultures were diluted 100 times. Mating was interrupted by T6 treatment and blending. Open circles, *proB*$^+$ recombinants obtained from G11 × D11; filled circles *proB*$^+$ recombinants obtained from G11 × D31 (with *ampAB*).

Fig. 2. Kinetics for the entrance of *proB*$^+$ in crosses with *ampA* in the donor and *ampAB* in the recipient. Cultures of G11a1 and D31 were mixed at zero time and divided into two parts. After 5 min one portion was diluted 100 times (filled circles); the other was kept undiluted (open squares). Mating was interrupted by T6 treatment and blending. Samples from the diluted culture were plated without further dilution while samples from the undiluted mating mixture were then diluted 100 times before plating.

(iv) *Characterization of some ampicillin resistant strains using bacteriophages*

The results from the crosses in the previous section (Figs. 1–3) are consistent with the interpretation that *ampAB*, and probably also *ampA* produce changes

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in the bacterial surface, which in turn produce pairing difficulties during mating. It is known that the pair formation involves F-pili and that these structures are also involved in the growth of the male specific RNA phages (see review by Brinton, 1967). We therefore, tested the ability of MS2 to produce plaques on some of our resistant strains. T4 and T5 were used as controls. In order to investigate the derivatives of D1, the F-factor from the reverted strain B8 (Broda, 1967) was

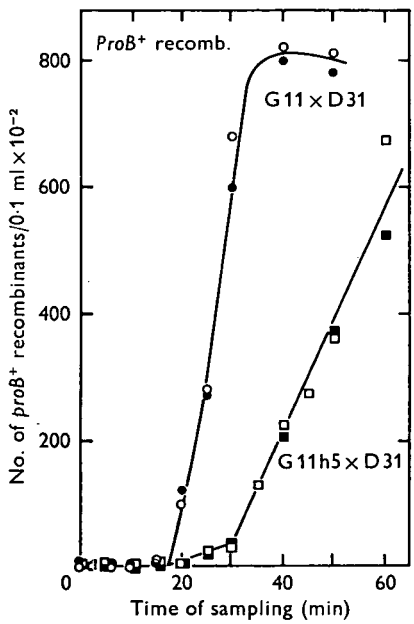

Fig. 3

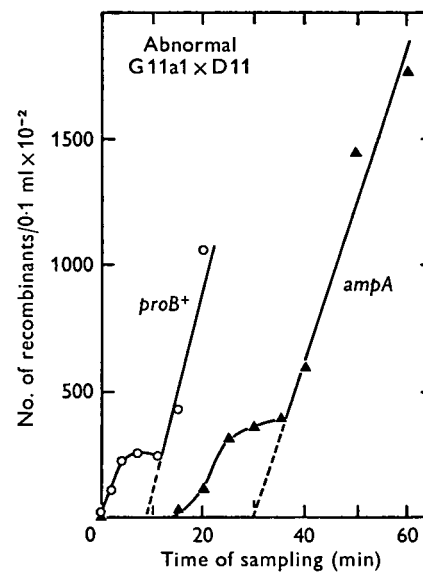

Fig. 4

Fig. 3. Kinetics for the entrance of *proB*+ (open symbols) in crosses with *ampAB* in both donor and recipient. Simultaneous selection for *proB*+ and *metB*+ (filled symbols). With G11h5 x D31 (squares) no T6 killing was used (cross 9 in Table 5); in the control experiments with G11 x D31 (circles) T6 was used to kill G11 (cross 4 in Table 5). The mating technique was the usual one described in Materials and Methods.

Fig. 4. Example of abnormal kinetics observed with derivatives of Hfr Cavalli. G11a1 x D11 with selection for *proB*+ (open circles) and *ampA* (filled triangles) recombinants. Mating was interrupted by 100-fold dilution and blending.

introduced into D21 and D31, thus producing the strains D21F+ and D31F+. The results of these experiments are given in Table 6 in the form of efficiency of plating (EOP) of the three phages tested. Phages MS2 showed difficulties in growing on three strains, namely AB311a1, AB311t6 and G11h5. Experiments with log. phase cultures showed that MS2 can grow on G11h5 but the increase in titre was less than 10% of the phage production obtained with G11a1. Table 6 shows that with other strains and phages only minor variations were obtained in the EOP. However, T4 consistently gave a reduced number of plaques on

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strain G11e1. For several of the strains plating of MS2 on sublethal concentrations of ampicillin also reduced plaque formation.

(v) *Transduction of ampicillin resistance*

We have earlier demonstrated that *ampA* could be transduced into sensitive recipients and that no cotransduction was obtained between *ampA* and *ampB* when G11e1 was used as donor (Eriksson-Grennberg *et al.* 1965). If our hypothesis that D31 contains a transposition of *ampA* was correct, cotransduction of

Table 6. *EOP for some phages on strains with different amp genes*

	Suggested genotype at		Phage		
Strain	Site 1	Site 2	MS2	T4	T5
AB311a1	*ampA*	+	< 0·01	1·0	1·0
AB311t6	*ampAB*/++ heterogenote		< 0·01	0·9	1·0
G11a1	*ampA*	+	1·0	1·0	1·0
G11e1	*ampA*	*ampB*	0·8	0·4	0·8
G11h5	—	*ampAB*	< 0·01	0·7	0·8
D21F+	*ampA*	+	0·8	1·0	0·9
D31F+	—	*ampAB*	0·9	1·0	1·0

EOP (efficiency of plating) was defined as the ratio between the plaque count obtained with a given strain and with G11a1. The phages (150–300 plaque forming units) were mixed in soft agar with the bacteria to be tested and poured on LA plates with $2{\cdot}5 \times 10^{-3}$ M-$CaCl_2$. The results are mean values obtained in three or more experiments with at least two different clones of bacteria. Plates with MS2 were incubated at 42 °C for 4–5 h, for T4 and T5 at 37 °C overnight.

Table 7. *Transduction of ampicillin resistance into the sensitive strain AB311*

Expt.	Donor	Frequency of *ampA* clones	Number of *ampA* transductants	Percentage with $75 < r < 100$
1	D3	7×10^{-7}	135	16
	D2	1×10^{-7}	15	0
	None	$< 10^{-8}$	0	—
2	D3	3×10^{-7}	51	90
	D2	1×10^{-7}	4	0
	G11e1	6×10^{-7}	33	0
	None	$< 10^{-8}$	0	—

In Expt. 2 all transductants were tested for *thr*, *leu* and *lac* markers of the recipient.

ampA and *ampB* could be expected if the two genes are sufficiently close. In preliminary experiments stocks of P1 were grown on G11h5 and D3 and tested on several recipients. Reasonable numbers of transductants were obtained only when using D3 as donor and AB311 as recipient, and AB311t6 was isolated in such an experiment. Table 7 gives the results of two other experiments in which transductants were selected on LA plates with D,L-ampicillin concentrations of 20 and 75 μg/ml. P1 grown on D2, on G11e1 and the spontaneous mutation level were used as controls. With D3 as donor, transductants were obtained only on 20 μg/ml with a frequency of about 10^{-7} which is slightly lower than previously

found for *ampA*. The transductants were purified and replicated on plates with D,L-ampicillin concentrations of 50, 75 and 100 μg/ml. It was found that several of the transductants obtained with D3 as donor grew on 75 μg/ml but none grew on 100 μg/ml. This is consistent with other observations which indicate that AB311 is more sensitive to ampicillin than are D11 or G11. Six of the most resistant transductants were tested by single-cell-colony formation on ampicillin and found to be of the amp-50 type. None of the fifteen transductants obtained with D2 or G11e1 as donor grew on plates with more ampicillin than 20 μg/ml. The data for the $75 < r < 100$ transductants given in Table 7 may, however, be uncertain since these strains were found to be unstable and to lose their resistance. Tests for sensitivity to P1 showed that all of the purified *ampAB* transductants were immune to superinfection with P1.

4. DISCUSSION

(i) *The mapping and the genetic constitution of ampAB strains*

There are two principle difficulties associated with the interpretation of crosses which involve a normal donor and a recipient with an assumed transposition or other type of aberration: (1) With two non-homologous chromosomes it cannot be predicted how pairing and recombination will proceed. (2) A transposition can be regarded as the result of an intra-chromosomal recombination and can thus be expected to be reversed by recombinational processes in general. It could therefore be possible that some of the recombinants in Tables 2–5 do not represent recombination between the donor and the recipient (inter-chromosomal recombination) but rather result from a reversed transposition.

Positive evidence for the hypothesis that D31 carries a transposition comes only from cross 1 and 2 (Table 2) and from the transduction experiments (Table 7). Since in cross 2 the same allele of *ampA* was present in both AB311a1 and D31, the occurrence of sensitive recombinants provides grounds for concluding that *ampA* did not have the same position in both of the strains. However, sensitive recombinants could also be expected if a gene exists which can decrease the resistance of another gene and if no segregation of the two genes causing resistance had occurred.

The transduction of the *ampAB* segment indicates that in D3 the distance between *ampA* and *ampB* must be smaller than the 2·0 min considered the maximum transducible length (Taylor & Trotter, 1967). This close linking is not fully consistent with the relatively high frequency observed for segregation of *ampA* and *ampB* in crosses 1 and 2. It is known that transduction between non-homologous chromosomes often gives rise to heterogenotes which carry a non-integrated and defective prophage of P1 (see Hayes, 1964). The finding that our *ampAB* transductants were unstable is therefore consistent with the transposition hypothesis but does not in itself constitute much support for this hypothesis.

The number of resistant classes obtained among the recombinants (Table 4) is an important fact in the interpretation of the crosses. Here the identification of the genes by resistance determinations is critical. However, even admitting fairly

large errors it seems difficult to reduce the number of classes from 5 to 4, which would be consistent with a simple two-gene system. The assumption of a three-gene system would require that some of the classes of recombinants are indistinguishable by our resistance tests. That additional genes for ampicillin resistance exist is indicated by the fact that we have recently isolated mutants which differ from all strains described here.

If D31 had a conventional genetic structure the crosses in Table 2 would indicate that *ampB* and perhaps also the *ampAB* segment were located somewhere in the region between *proB* and *trp* as showed in Fig. 5. However, this interpretation becomes somewhat uncertain since Hfr strains injecting clock-wise did not give the same segregation pattern as obtained with strains injecting counter clock-wise. Other types of experiments are therefore needed for a complete characterization of the *ampAB* mutation.

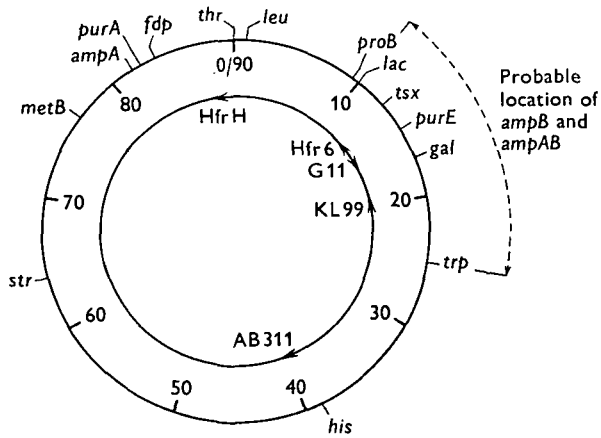

Fig. 5. Map of the *E. coli* chromosome with the *amp* genes, other relevant markers and the Hfr strains used. The gene order around 10 min is according to Taylor & Dunham Trotter (1967). For abbreviations see Table 1.

(ii) *The gene products of ampA and ampB and their physiological roles*

Strain D31 contains the *ampA1* allele of G11a1 and the same penicillinase should therefore be expected to be present in both strains. This enzyme has now been purified from both strains and found to be the same as judged by immunological and enzymatic characterization (Lindström & Boman, 1968). However, in minimal medium D31 contains about 3 times more penicillinase than G11a1. Results presented in the preceding paper (Nordström *et al.* 1968) indicate that *ampB* is a 'modifying gene' which can influence both *ampA* and R-factors. Recently we have also found that growing and non-growing cells of D31 differ considerably in their ability to hydrolyze ampicillin (Burman, Nordström & Boman, 1968). It is therefore difficult to be certain that the penicillinase found in D31 is the full explanation for the resistance of this strain.

It has been suggested that penicillinases could be enzymes participating in cell wall biosynthesis (Eriksson-Grennberg *et al.* 1965; Boman *et al.* 1967; Pollock, 1967). The pairing difficulties during mating with *ampAB* strains (Figs. 1–3) as well as the growth difficulties for MS2 which were observed with G11h5 are consistent with the expectation that mutations affecting cell-wall enzymes may produce a changed cell surface. Nordström & Burman (1968) have recently found that *ampB* gives an increased sensitivity to osmotic shock and increased sensitivity to cycloserine. Both these findings indicate that *ampB* weakens the cell wall. The evidence summarized by Brinton (1967) shows that F-pili are involved in both conjugation and growth of RNA phages. It can thus be suggested that *ampAB* controls a product needed either for the formation of F-pili or for their attachment to the bacterial surface. Figure 3 shows that at the end of the mating time G11h5 gave nearly the same number of recombinants as were obtained with G11. It therefore seems unlikely that the presence of *ampAB* gives a reduced number of F-pili and that this is the sole explanation for the abnormalities observed with G11h5. If *ampAB* has a qualitative effect on F-pili or their attachment site, this could be only one of several changes produced. It cannot be ruled out that the poor growth of MS2 was due to an incomplete release of mature phage rather than to a change in the F-pili.

(iii) *The evolutionary aspects of ampicillin resistance*

There are two evolutionary aspects of the *ampAB* system. The first concerns short-term evolution and the function of the wild type alleles of *ampA* and *ampB* discussed in the previous section.

The second aspect deals with the origin of R-factors carrying ampicillin resistance. Smith (1967) recently found evidence that R-factors existed as early as 1946, which is before the common use of antibiotics. Watanabe (1963, 1967) has suggested that R-factors are of chromosomal origin. This view is supported by the demonstration of Harada, Kameda, Suzuki, Shigehara & Mitsuhashi (1967) that tetracycline resistance carried by an R-factor was integrated into the host chromosome and by the finding of Pearce & Meynell (1968) that a mutant of *R1* showed a specific affinity for the chromosome. It is also interesting that the locations of chromosomal resistance to chloramphenicol (Reeve & Suttie, 1968) and tetracycline (Reeve, 1968) are relatively near the probable site of *ampAB*. If R-factors originate from chromosomal genes it seems likely that, as a first step, resistance genes are brought together in a certain area of the chromosome. A transposition of the *ampA* gene could therefore be reasonable from an evolutionary point of view. Our evidence that chromosomal ampicillin resistance is mediated by genes affecting the cell surface and the fact that infectivity has to be associated with surface information are consistent with the suggestion that R-factors have evolved from chromosomal genes concerned with the cell surface.

SUMMARY

The first two steps towards increasing ampicillin resistance in *Escherichia coli* concern the genes *ampA* and *ampB* which are located at least 20 min from each other (Eriksson-Grennberg *et al.* 1965 and the two preceding papers). This paper describes a third class of *ampA*-containing mutants (designated *ampAB*) which are resistant to D,L-ampicillin concentrations of 100 μg/ml. When such as F^- strain (D31) was crossed to different Hfr strains analysis of the trp^+ and $proB^+$ recombinants indicated that resistance genes were located between *trp* and *proB*. Altogether five classes of recombinants were produced but only the two genes *ampA* and *ampB* were recovered. One interpretation suggested is that the resistance of D31 is due to the presence of *ampA* and *ampB* and a chromosomal aberration by which *ampA* was moved to a position near *ampB*. It was possible to transduce both intermediate levels of resistance as well as the full resistance of an *ampAB* donor strain, but the strains produced were unstable. In crosses the presence of *ampAB* in the recipient reduced the number of recombinants by reducing the number of stable pairs. In an Hfr strain *ampAB* was shown to give rise to additional difficulties in establishing the cell contact during mating. Some ampicillin resistant mutants also showed decreased ability to propagate the RNA phage MS2.

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