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Resistance of *Escherichia coli* to penicillins

II. An improved mapping of the *ampA* gene

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1. INTRODUCTION

The development of penicillin resistance in Gram positive bacteria was early found to be a multistep process giving gradually increased resistance for each of several consecutive mutations. This pattern also applies to ampicillin resistance in *Escherichia coli* (Eriksson-Grennberg, Boman, Jansson & Thorén, 1965). The first step involves a gene (*ampA*) which after a single mutation provides resistance to a D,L-ampicillin concentration of 10 μg/ml. Genetic analysis showed that the *ampA* locus maps between *argH* and *pyrB*.* As a second step towards increasing resistance the bacteria acquire a mutation in a modifying gene, *ampB*, (Nordström, Eriksson-Grennberg & Boman, 1968) located between *proB* and *trp* (Boman, Eriksson-Grennberg, Normark & Matsson, 1968). An *ampA* containing mutant resistant to a D,L-ampicillin concentration of 100 μg/ml was found to have a complex genetic character. The data were interpreted as indicating that a chromosomal rearrangement had moved *ampA* from the *argH-pyrB* region to a position near *ampB* (Boman *et al.* 1968). This hypothesis, together with the need for strains carrying only *ampB*, prompted the more detailed mapping of *ampA* given in this communication. Some preliminary results have been presented at meetings (Boman, Eriksson-Grennberg, Földes & Lindström, 1967; Eriksson-Grennberg, 1968).

2. MATERIALS AND METHODS

(i) *Organisms*

All bacterial strains used were *E. coli* K12; their properties and origin are shown in Table 1. *AmpA* from G11a1 was transduced to Hfr Reeves 1 giving R12. Strain KG25 is a *thr*$^+$*leu*$^+$T4-resistant recombinant from a cross between KA66 and a T4-resistant derivative of PA373. The strains PA256 and PA373 did not grow on either citrulline or ornithine and should therefore carry the locus *argH* in the cluster *argBCEH* at 77–78 min. *ArgH* was earlier designated *argF* in the map of Taylor & Thoman (1964) and in our previous paper (Eriksson-Grennberg *et al.* 1965). For transduction experiments the phage Plbt was used (Gross & Englesberg, 1959).

* Capital letters refer to the chromosomal map of Taylor & Trotter (1967).

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

Strain	Reference	Sex	Ampicilline genotype		Response to *str*	Other relevant markers
			Site 1	Site 2		
G11	Stent & Brenner (1961)	Hfr	+	+	s	*metB*, *ilv*
G11a1	Eriksson-Grennberg *et al.* (1965)	Hfr	*ampA1*	+	s	*metB*, *ilv*
G11e1		Hfr	*ampA1*	*ampB1*	s	*metB*, *ilv*
P4xa2		Hfr	*ampA2*	+	s	*metB*
P4xa21		Hfr	*ampA2*	+	s	
Hfr Reeves 1	R. Hill	Hfr	+	+	s	*metB*
R12	See Materials and Methods	Hfr	*ampA1*	+	s	*metB*
KA66	I. E. Mattern	Hfr	+	+	r	*his*, *uvrA*
Q11	Fraenkel (1967)	Hfr	+	+	s	*fdp*
KG20	Eriksson-Grennberg *et al.* (1968)	F+	*ampA1*	+	r	*his*, *argH*, *pro*
KG20e1		F+	*ampA1*	*ampB3*	r	*his*, *argH*, *pro*
KG25	See Materials and Methods	F−	+	+	r	*his*, *argH*, *metA*, *uvrA*, *T4*r
PA256	R. Lavallé	F−	+	+	r	*his*, *argH*, *purA*, *pro*
PA373		F−	+	+	r	*his*, *argH*, *metA*, *thr*, *leu*
PA2004		F−	+	+	r	*his*, *pyrB*, *thr*, *leu*

* Abbreviations: amp, ampicillin; arg, arginine; fdp, fructose diphosphatase; his, histidine; *ilv*, isoleucine-valine; leu, leucine; met, methionine; pro, proline; pur, purine; pyr, pyrimidine; str, streptomycin; thr, threonine; uvr, UV-sensitivity; *T4*r, resistance to phage T4; s, sensitivity; r, resistance. Capital letters after some of the symbols refer to the nomenclature of Taylor & Trotter (1967).

(ii) *Materials*

Ampicillin with D- and L-epimers in the ratio 6:4 and 4:6 and pure D-epimer was kindly donated by Astra, Södertälje, Sweden. Streptomycin sulfate was a gift from Kabi, Stockholm, Sweden. Nylon microculture containers were obtained from Elesa, Milano, Italy.

(iii) *Media*

Minimal medium was made from the basal medium E of Vogel & Bonner (1956) supplemented with 0·2% glucose, thiamine (1 μg/ml) and with the required amino acids (25 μg/ml of the L-epimer). For PA256 and descendants it was necessary to enrich the minimal medium with a solution of eleven additional amino acids and with a mixture of vitamins. The amino acid solution contained 0·5 g of DL-alanine, L-asparagine, L-lysine, DL-phenylalanine, glycine, DL-serine, 0·1 g of L-cysteine, L-tyrosine, and L-glutamine and 1·0 g of DL-valine and DL-isoleucine in 100 ml of water. Of this solution 0·5 ml was added to 100 ml medium. The vitamin solution (Gensone, personal communication) contained per litre: 200 mg Ca-pantothenate, 250 μg biotin, 100 mg nicotinic acid, 100 mg thiamine, 100 mg riboflavin, 500 mg inositol, 50 mg *p*-aminobenzoic acid and 100 mg pyridoxine. This mixture was sterilized by autoclaving. A separate solution of folic acid (400 μg/ml) was sterilized by filtration. One ml of the vitamin mixture was added to 100 ml medium. When required, adenine was added to a concentration of 10 μg/ml. Plates were solidified with 1·5% Difco agar. Transduction and conjugation experiments were done in the LB medium of Bertani (1951). *AmpA* recombinants and transductants were scored on LB medium solidified with 1·5% agar (= LA plates) supplemented with 10 μg of ampicillin per ml. Streptomycin was used for counterselection; the plates were supplemented with 100 μg of streptomycin sulphate per ml.

(iv) *Mating conditions and selection of UV-resistant recombinants*

The conjugation experiments were done as described by Eriksson-Grennberg *et al.* (1965) except that LB medium was used. Strain PA256 did not grow in the cross-medium previously used. UV-resistant recombinants were selected as described by Van de Putte, Van Sluis, Van Dillewijn & Rörsch (1965).

(v) *Transduction experiments*

In the beginning of these investigations *Shigella dysenteriae* strain Sh15 was used as an indicator for P1. Later *Escherichia coli* strain C1055 was used, both were obtained from Dr G. Bertani, Stockholm. Phage stocks were prepared by the agar layer method of Adams (1959). However, with slow growing bacteria the titres obtained were only 10^8 plaque forming units/ml. About 100 times higher titres were obtained by the following procedure which in turn is a modification of a description circulated by Dr Helling (private communication). Overnight cultures of bacteria were centrifuged and concentrated 10 times in fresh LB medium containing $2{\cdot}5 \times 10^{-3}$ M-$CaCl_2$ before they were added to phages in soft agar. The mixtures were poured on Ca^{2+}-containing LA plates, which were no older than 24 h. After 6 h

3 ml of distilled water was added to each plate and the soft agar layers were scraped off. The agar debris was removed by centrifugation. The supernatant was mixed with chloroform and stored overnight at 4 °C. Next day the phage stock was again centrifuged to remove a small amount of precipitate.

Transduction experiments were performed according to the following procedure. An overnight culture grown in LB containing 0·01 M-$CaCl_2$ was divided into two 10 ml parts and centrifuged. The cultures were concentrated 10 times. Transducing phages were added to one of the cultures to about one phage per bacterium. The other culture without phases was the control. To both cultures 0·01 ml of 0·01 M-$CaCl_2$ was added and the volumes of the cultures were adjusted to 1 ml with 0·9 % NaCl. To allow time for phage adsorption and DNA injection both cultures were incubated for 30 min at 37 °C. After incubation the cells were diluted with 10 ml of 0·5 % NaCl, centrifuged and resuspended in 10 ml of LB. To allow time for phenotypic expression the bacteria were grown on a rotary shaker at 37 °C for 3–4 h and washed twice with 10 ml of NaCl before spreading on plates with selective media. PA256 transductants were picked, on rich media after 40 h, on minimal media after 50 h, purified on the selection media and tested by replica plating.

3. RESULTS

(i) *Conjugation experiments*

In a previous paper it was shown that the *ampA* locus is located between *argH* and *pyrB* (Eriksson-Grennberg *et al.* 1965). This conclusion was based on crosses with two different ampicillin resistant Hfr strains which both transferred chromosomal markers in the order: *O-pro-thr-ilv*. The *ampA* location has now been confirmed using an ampicillin resistant derivative of Hfr Reeves 1, R12, which injects the chromosome in the reverse direction with *ilv* as an early marker. Two such crosses are given in Figs. 1 and 2. The results show that the genes enter in the order: *argH*, (*purA ampA*) and *pyrB*. *AmpA* and *purA* seem to be closely linked. This was further demonstrated by an analysis of the recombinants from this cross as well as from another similar experiment. Table 2 shows 50 % linkage between *argH* and *ampA* and 96 % linkage between *purA* and *ampA*.

A gene providing sensitivity to ultraviolet light, *uvrA*, has been found to be located between *metA* and *pyrB* (Howard-Flanders, Boyce & Theirot, 1966). To investigate the order of *ampA* and *uvrA*, the F^- and *uvrA* strain KG25 was crossed with the *ampA*-containing Hfr strain P4xa21, which injects the chromsome in the order *O-proB-thr-metB*. Figure 3 shows that *ampA* was transferred somewhat earlier than $uvrA^+$. $UvrA^+$ recombinants, obtained after 30 and 35 min of mating respectively, were picked and purified. *AmpA* recombinants were also picked and purified from the same times. 19 of 22 recombinants selected for $uvrA^+$ after 30 min of mating were found to have received the *ampA* locus. Among the recombinants selected for *ampA* only 14 of 49 had received the gene $uvrA^+$. For recombinants obtained after 35 min of mating the corresponding figures were 38 of 42 and 18 of 50, respectively.

(ii) *Transduction experiments*

Previous work (Eriksson-Grennberg *et al.* 1965) showed that the *ampA* gene was expressed without any significant lag period. The frequency of transduction of *ampA* was 5×10^{-7}. In preliminary transduction experiments with PA256 as recipient very few *ampA* transductants were obtained. For this reason the phenotypic expression of *ampA* in PA256 was investigated. Figure 4 shows that no transductants were obtained during the first 2 h. The frequency then increased to

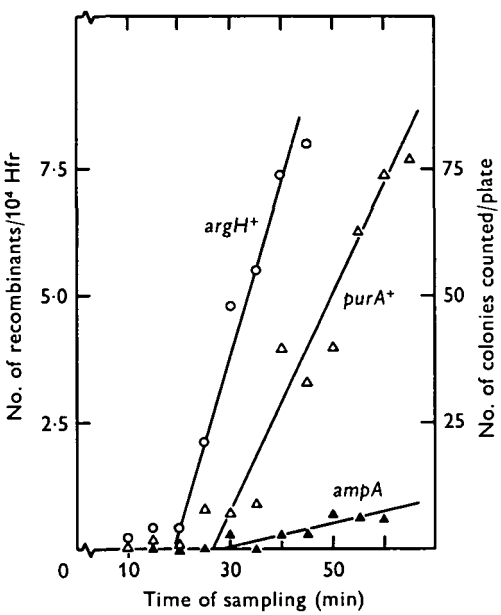

Fig. 1

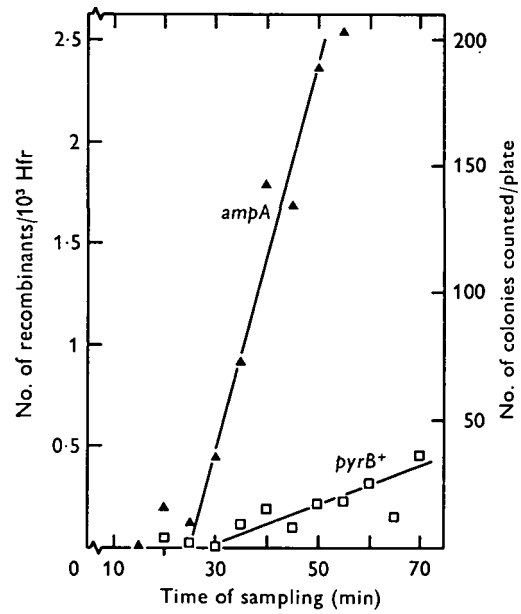

Fig. 2

Fig. 1. Kinetics of chromosome transfer by donor strain R12 to recipient strain PA256. *ArgH*$^+$ recombinants (○), *purA*$^+$ recombinants (▲) and *ampA* recombinants (▲).

Fig. 2. Kinetics of chromosome transfer by donor strain R12 to recipient strain PA2004. *PyrB*$^+$ recombinants (□) and *ampA* recombinants (▲).

Table 2. *Analysis of recombinants in cross R12 × PA256*

PurA$^+$/*str-r* recombinants				*ArgH*$^+$/*str-r* recombinants			
	No. of recombinants				No. of recombinants		
Unselected markers	Expt. 1	Expt. 2	Mean frequency	Unselected markers	Expt. 1	Expt. 2	Mean frequency
argH$^+$; *ampA*	132	295	0·82	*purA*$^+$; *ampA*	177	264	0·46
argH; *ampA*	28	43	0·14	*purA*; *ampA*	21	21	0·04
argH$^+$; *ampA*$^+$	2	3	0·01	*purA*$^+$; *ampA*$^+$	4	8	0·01
argH; *ampA*$^+$	5	9	0·03	*purA*; *ampA*$^+$	137	324	0·49

Expt. 2 is the same experiment as shown in Fig. 1. Recombinants were picked at different times with the beginning after 30 min of mating, when *ampA* has begun to enter.

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about 5×10^{-8} but it did not reach the efficiency previously obtained. The same pattern was found irrespective of whether G11a1 or G11e1 was used as donor. Controls, to which no phages were added, gave no colonies. In later transduction experiments 3–4 h were allowed for phenotypic expression.

Lavallé (personal communication) and Howard-Flanders *et al.* (1966) have found the genes *purA* and *uvrA*, respectively, to be located between *metA* and *pyrB*

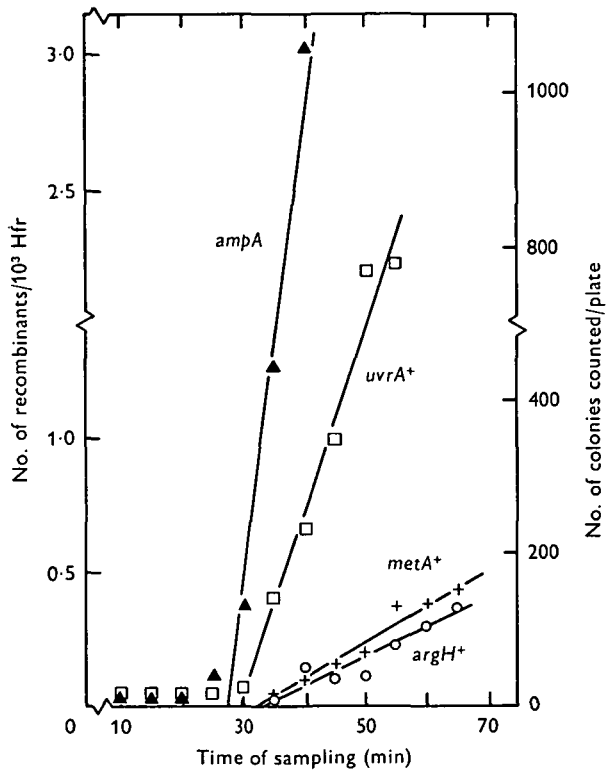

Fig. 3. Kinetics of chromosome transfer by donor strain P4xa21 to recipient strain KG25. *AmpA* recombinants (▲), *uvrA*⁺ recombinants (□), *metA*⁺ recombinants (+) and *argH*⁺ recombinants (○).

and Fraenkel (1967) has mapped *fdp* to the same region. The purpose of the present set of transduction experiments was to confirm the gene order indicated from the crosses described above and to insert *fdp*, *purA* and *ampA* in the correct positions in the interval *argH* to *pyrB*.

Table 3 shows that the cotransduction between *purA*⁺ and *ampA* was about 40% with selection for *purA*⁺ (Expts. 1, 4 and 5). When selected for *ampA* the cotransduction with *purA*⁺ was 75% (Expts. 2 and 3). However, it was not possible to cotransduce *ampA* and *purA* using selection for *ampA*. *PurA* and *ampA* were also cotransduced with *fdp* (Expts. 11–13). No cotransduction was found between *ampA* and *argH*, *metA*, *uvrA* or *pyrB* (Expts. 6–10). Preliminary trans-

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duction experiments which indicated cotransduction between *metA* and *ampA* were communicated to Dr Taylor (mentioned by Taylor & Dunham Trotter, 1967). However, it has not been possible to repeat this cotransduction.

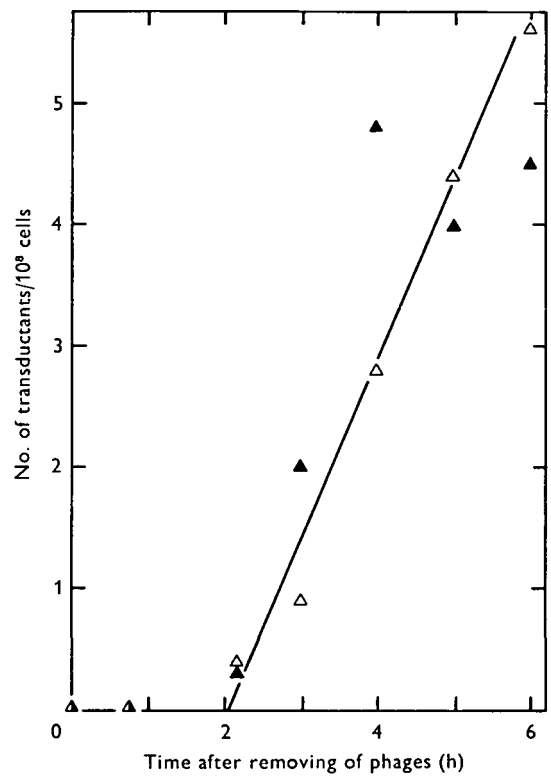

Fig. 4. Phenotypic expression of the *ampA* gene after transduction from the donor strains G11a1 (▲) and G11e1 (△) to the recipient strain PA256. The phage-treated bacteria were incubated in LB at 37 °C, samples were removed and concentrated 4 times before spreading on plates containing D-ampicillin (10 μg/ml) and streptomycin (100 μg/ml). The number of bacteria was measured during the incubation on a Klett–Summerson colorimeter. 100 Klett units correspond to about 4×10^8 viable cells/ml.

4. DISCUSSION

From the conjugation experiments shown in Figs. 1–3 it can be concluded that the *ampA* locus is located between *pyrB* and *uvrA*. The gene order between *purA* and *ampA* can be determined from the analysis of the recombinants from the conjugation experiment shown in Fig. 1 as well as from a similar one (see Table 2). Among the $purA^+$ recombinants the rarest combination of unselected markers was $argH^+$ $ampA^+$; among the $argH^+$ recombinants the rarest combination was $purA^+$ $ampA^+$. Assuming these two groups to result from double cross-overs in the region, the most likely gene order is *argH-ampA-purA*. From Fig. 3 and the analysis of the *uvrA* and *ampA* recombinants it follows that the gene order is *argH-uvrA-ampA-purA*.

Table 3. P1bt *mediated transduction of genes in the* argF-pyrB *region*

Expt. no.	Donor	Recipient	Selection	No. of transductants scored	Genes cotransduced	% cotransduction
1	G11a1	PA256	*purA*$^+$	259	*purA*$^+$-*ampA*	42
2	G11e1	PA256	*ampA*	36	*ampA*-*purA*$^+$	70
3	P4xa2	PA256	*ampA*	170	*ampA*-*purA*$^+$	82
4	G11	KG20e1	*purA*$^+$	46	*purA*$^+$-*ampA*$^+$	35
5	PA373	KG20	*purA*$^+$	33	*purA*$^+$-*ampA*$^+$	54
					purA$^+$-*metA*	0
6	G11a1	PA2004	*ampA*	365	*ampA*-*pyrB*$^+$	0
7	P4xa2	PA373	*ampA*	50	*ampA*-*metA*$^+$	0
					ampA-*argA*$^+$	0
			metA$^+$	137	*metA*$^+$-*ampA*	0
					metA$^+$-*argH*$^+$	40
8	G11a1	PA373	*ampA*	181	*ampA*-*metA*$^+$	0
9	G11a1	KA66	*ampA*	395	*ampA*-*uvrA*$^+$	0
10	P4xa2	KA66	*ampA*	188	*ampA*-*uvrA*$^+$	0
11	G11a1	Q11	*fdp*$^+$	16	*fdp*$^+$-*ampA*	6
12	P4xa2	Q11	*fdp*$^+$	116	*fdp*$^+$-*ampA*	5
13	Q11	KG20	*purA*$^+$	151	*purA*$^+$-ampA$^+$	46
					purA$^+$-*fdp*	14
					purA$^+$-*ampA*$^+$-*fdp*	2

The experiments in Table 3 show that the frequency of cotransduction of *ampA* and *purA*$^+$ was higher than for other genes. With selection for *ampA*, the cotransduction was 70–80% (Expts. 2 and 3), while selection for *purA*$^+$ gave 40–50% cotransduction (Expts. 1, 4 and 5). The fact that no cotransduction was found between *ampA* and *purA* may be due to a lower viability of the purine requiring cells. It may also depend on differences in the phenotypic expression of the two genes. Figure 4 shows that in PA256 the time required for expression of *ampA* is about 3–4 h. This result differs from the phenotypic expression of *ampA* in strain AB325(λL26) (Eriksson-Grennberg *et al.* 1965). Since purine requirement decreases growth rate, it seems likely that the *purA* cotransductants possibly obtained have been overgrown by *purA*$^+$ cells and therefore not detected. These differences in growth rate and phenotypic expressions can account for the fact that selection influenced the cotransduction frequency between *ampA* and *purA*$^+$.

For the above reasons, care must be exercised in the use of the cotransduction data (Table 3) for estimating map distances. Taking the largest cotransducible segment to 1·5 min (Taylor & Thoman, 1964) the combined data was used to compile the map shown in Fig. 5. Table 3, experiment no. 13 shows a three factor cross with selection for *purA*$^+$. The cotransduction observed was for *purA*$^+$ and *ampA*$^+$ 46%, for *purA*$^+$ and *fdp* 14%, and for *ampA*$^+$ and *fdp* 2%. Assuming that the expressions of *ampA*$^+$ and *fdp* were similar, the results show that *fdp* and *purA* are more closely linked than *fdp* and *ampA*. Fraenkel (1967) has reported 64% cotransduction between *pyrB* and *fdp* and Howard-Flanders *et al.* (1966) have shown 4% cotransduction between *uvrA* and *metA*. No cotransduction was found between *ampA* and *metA*, *uvrA* or *pyrB* (Expts. 6–10). Despite the absence

of cotransduction between *uvrA* and *ampA*, the results in this paper indicate that the probable gene order is *argH-metA-uvrA-ampA-purA-fdp-pyrB*. Both conjugation and transduction experiments show that two independent mutations, *ampA1* and *ampA2* as well as the *amA1* transduced into strain R12, are located at 82 min. This is emphasized since data presented in a companion paper (Boman *et al.* 1968) indicate that an alternative location is possible at least in the F⁻ strain D31.

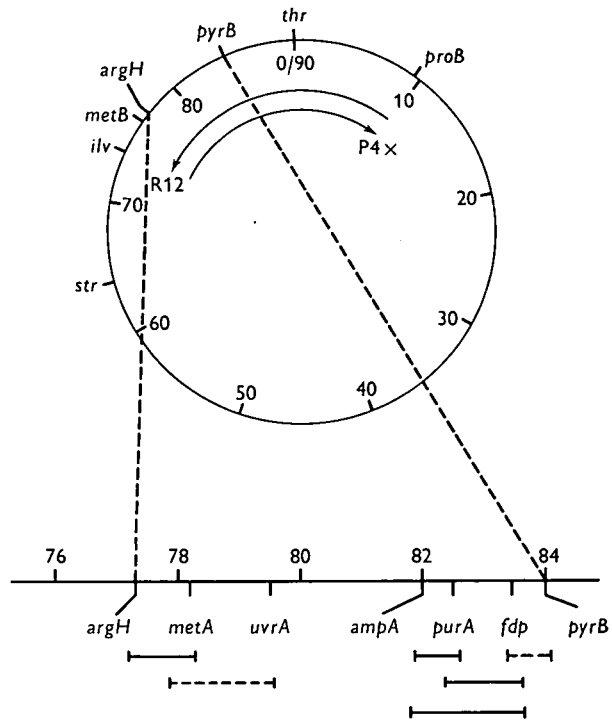

Fig. 5. Genetic map of the *E. coli* chromosome with an enlargement of the *argH-pyrB* region. Time indications and markers according to Taylor & Dunham Trotter (1967). The origins and order of injection for Hfr strains used are indicated by arrows. Cotransduction data from this paper (⊢—⊣) and from Howard-Flanders *et al.* (1966) & Fraenkel (1967) (⊢- - -⊣).

Preliminary work with whole cells indicated that the *ampA* gene gives rise to penicillin-β-lactamase activity (Eriksson-Grennberg *et al.* 1965). This enzyme has now been purified (Lindström & Boman, 1968), both from G11a1 and the wild type strain G11. The two proteins were indistinguishable by immunological tests. Further work is however needed to clarify whether the *ampA* gene is the structural gene for this penicillinase.

SUMMARY

The first mutation step towards ampicillin resistance in *Escherichia coli* occurs in the *ampA* gene, and gives resistance to a D,L-ampicillin concentration of 10 μg/ml. Using interrupted conjugation and transduction experiments *ampA* was

found to be located at 82 min on the time scale of Taylor & Thoman (1964). A number of adjacent markers were studied and the probable gene order of the *ampA* region was found to be *argH-metA-uvrA-ampA-purA-fdp-pyrB*. Two independent alleles of *ampA* were cotransduced with *fdp*$^+$ and *purA*$^+$. The phenotypic expression of *ampA* in a *purA* strain has been investigated.

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