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Escherichia coli K-12 Mutants Hyperproducing Chromosomal Beta-Lactamase by Gene Repetitions

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Escherichia coli K-12 ampicillin-resistant mutants hyperproducing chromosomal β-lactamase arose spontaneously from strains carrying *ampA1 ampC*$^+$. Such mutants were found even in a *recA* background. Two Amp^r-100 strains were analyzed genetically. The Amp^r-100 resistance level of both strains could be transduced by direct selection for ampicillin resistance. Several classes of ampicillin-resistant transductants were found that differed from one another in the β-lactamase activity and the ampicillin resistance mediated by an *ampA1 ampC*$^+$-carrying strain. The data suggested that β-lactamase hyperproduction was due to repetitions of the chromosomal *amp* genes. The size of the repeated region was calculated from cotransduction estimates, using the formula of Wu (Genetics **54**:405-410, 1966), and was found to be about 1 min in one strain and 1.5 min in the other. Second-step Amp^r-400 mutants were isolated from an Amp^r-100 strain. The resistance of these mutants was apparently also due to repetitions, each mediating a resistance to about 10 μg/ml. Mutants of wild-type strains that were moderately resistant to ampicillin also gave rise to intermediate-resistance classes, suggesting repetitions of the wild-type *amp* alleles. F′ factors hyperproducing chromosomal β-lactamase by gene repetitions were constructed. They mediated levels of ampicillin resistance comparable to that of naturally occurring resistance plasmids. The expression of β-lactamase hyperproduction was not affected by the presence of *ampA* and *ampC* alleles in *trans* and did not act in *trans* on the other alleles.

Wild-type *Escherichia coli* K-12 produces a small amount of a chromosomally mediated β-lactamase. The enzyme activity apparently does not contribute to the penicillin resistance of wild-type strains, which are resistant to ca. 1 to 2 μg of ampicillin per ml. It has been suggested, therefore, that the chromosomal β-lactamase fulfills another function in the cell (9). Ampicillin-resistant mutants of *E. coli* K-12 can be isolated at a low incidence. The first mutant allele studied was designated *ampA1* and was located at 93 min on the revised chromosomal map of *E. coli* (1, 13). Strains carrying the *ampA1* allele are resistant to 10 μg of ampicillin per ml and contain about 10 times the amount of β-lactamase found in wild-type strains. β-Lactamase purified from strains carrying the wild-type or *ampA1* allele showed no differences in enzymatic properties (24). Mutants defective in β-lactamase production have been obtained from *ampA1*-carrying strains. In two of these mutants the purified β-lactamase was biochemically altered, suggesting a lesion(s) in the structural gene, designated *ampC* (9). The structural gene *ampC* was recently mapped very close to *ampA*, the gene order being *ampC-ampA-purA*. The *ampA1* mutation was also found to be dominant in *cis* but not in *trans* (31). The accumulated data therefore suggest that *ampA* is the operator-promoter region for *ampC*.

Spontaneous as well as mutagen-induced mutants with enhanced ampicillin resistance have been isolated from *ampA1*-carrying strains (7). In many of these mutants the lipopolysaccharide portion of the outer membrane was altered (6, 7, 28). However, one ethyl methane sulfonate-induced mutant showed in addition an increased β-lactamase content (24). Thus, apparently genetic events other than the *ampA1* mutation may affect β-lactamase production. It is known that genetic duplications are rather common events in the *E. coli* chromosome (2, 16, 17, 22). In view of our earlier finding that ampicillin resistance and β-lactamase activity double in homozygous merodiploids (31), we hypothesized that mutants with duplications of the *amp* genes would be more ampicillin resistant. We thus isolated and studied a number of spontaneous mutants that appeared to contain several copies of the chromosomal *amp* genes.

MATERIALS AND METHODS

Organisms. *E. coli* K-12 strains used are listed in Table 1. *recA* derivatives of various strains were obtained in crosses with Hfr KL16-99 *recA*. The *recA* allele was scored by the ultraviolet sensitivity method of Clark and Margulies (11). The F′ factor F′1183 contains the β-lactamase genes of the highly ampicillin-resistant mutant TE01. It was constructed as follows. F′118 (*ampA*$^+$ *ampC*$^+$) was introduced into strain TE01. One diploid clone was grown in LB medium (see below) for several generations and was then crossed with strain SN01 (*ampA*$^+$ *ampC*$^+$ *recA*). Clones were scored as Ura$^+$ His$^+$ and as resistant to 100 μg of ampicillin per ml. The presence of F′ factors was tested by selecting for Ade$^+$ progeny in a mating on plates, with strain PA256 as a recipient.

Media and growth conditions. The minimal medium used was medium E (36) supplemented with 0.2% glucose, 1 μg of thiamine per ml, 100 μg of the L-isomer of each required amino acid per ml, and, when necessary, 25 μg of uracil per ml. The Casamino Acids medium contained basal minimal medium, 1.5% casein hydrolysate, 0.2% glucose, and 25 μg of uracil per ml when required. The complete medium was LB medium of Bertani (4) supplemented with medium E and 0.2% glucose. It was solidified with 1.5% agar (LA plate). Unless otherwise stated, the experiments were performed at 37°C. The bacteria were cultivated on a rotary shaker, and growth was recorded by optical density readings, using a Klett-Summerson colorimeter with a W66 filter.

Materials. α-Amino-benzylpenicillin (D-ampicillin) and benzylpenicillin (penicillin G) were kindly provided by AB Astra, Södertälje, Sweden. Streptomycin sulfate was donated by AB Kabi, Stockholm, Sweden. Lysozyme was from Sigma Chemical Co., St. Louis, Mo. Zulkowskys starch (used in β-lactamase determination) was from E. Merck AG, Darmstadt, Germany.

Determination of ampicillin resistance. Ampicillin resistance was carefully determined for single cells as previously described (9). Resistance was defined as the concentration level permitting 50% of the cells to form colonies. For testing the resistance of a large number of clones, a steel replicator was used (8).

Determination of β-lactamase activity. β-Lactamase activity was determined as previously described (31). One unit of β-lactamase was defined as the enzyme activity that hydrolyzed 1 μmol of benzylpenicillin per h in 0.05 M phosphate buffer (pH 7.4) at 37°C. Specific β-lactamase activity was expressed as units per milligram of protein. Protein was determined by the method of Lowry et al. (26), with bovine serum albumin as a standard.

Mating procedure. Conjugation experiments using Hfr or F′ strains as donors were performed as previously described (29, 30), except that 0.1 ml of the mating mixture was mixed directly with 5 ml of melted top agar and poured onto selective plates (27).

Transduction procedure. The transduction procedure with phage P1*clr*100*cml* was as described by Rosner (35). When ampicillin-resistant transductants were selected, the bacteria were suspended in LB medium and grown for 3 h before plating to allow for phenotypic expression. Transduction with phage P1*v* was performed as previously described (13).

Immunoelectrophoresis of β-lactamase. The

TABLE 1. *E. coli K-12 strains and their relevant characters*

Strain	Sex	Genotype or phenotype		Other relevant markers	Source, derivation, or reference
		ampA	*ampC*		
G11	Hfr	+	+	*metB ilv*	HfrC, G. Stent
G11a1	Hfr	1	+	*metB ilv*	*ampA1* transductant of G11
KL25	Hfr	+	+		B. Low (25)
SN06	Hfr	1	+		*ampA1* transductant of KL25
KL16-99	Hfr	+	+	*recA*	B. Low (25)
UM100	Hfr	1	8	*metB*	(9)
D21	F$^-$	1	+	*trp proA his rpsL*	H. G. Boman (7)
Q11	F$^-$	+	+	*fdp*	D. G. Fraenkel, see also reference 13
PA256	F$^-$	+	+	*pro his argF purA rpsL*	R. Lavallé, see also reference 13
Hlt14	F$^-$	1	12	*leu thr proA rpsL*	(9)
LA5	F$^-$	+	+	*pyrB thr leu his rpsL*	PA2004, R. Lavallé
LA51	F$^-$	1	+	*pyrB thr leu his rpsL*	*ampA1* transductant of LA5
SN01	F$^-$	+	+	*pyrB thr leu recA rpsL*	*recA* derivative of LA5 (31)
SN02	F$^-$	1	+	*pyrB thr leu recA rpsL*	*recA* derivative of LA51 (31)
SN01/F′118	F′	+/+	+/+	*pyrB thr leu recA rpsL*/F′118	(31)
SN01/F′1181	F′	+/1	+/+	*pyrB thr leu recA rpsL*/F′1181	*ampA1* on episome (31)
SN01/F′1182	F′	+/1	+/12	*pyrB thr leu recA rpsL*/F′1182	*ampA1* and the temperature-sensitive *ampC12* allele on episome (31)
HW01	F$^-$	Amp^r-100		*proA trp his rpsL*	Amp^r-100 mutant of strain D21
TE01	F$^-$	Amp^r-100		*pyrB thr leu his rpsL*	Amp^r-100 transductant, cross P1 (HW01) × LA51
TE13	F$^-$	Amp^r-100		*pyrB thr leu his rpsL*	Amp^r-100 mutant of LA51
SN01/F′1183	F′	Amp^r-100		*pyrB thr leu recA rpsL*/F′1183	Amp^r-100 phenotype of TE01 on episome
SN04	F$^-$	Amp^r-100		*pyrB thr leu recA rpsL*	*recA* derivative of TE01
SN05	F$^-$	Amp^{r}90		*pyrB thr leu recA rpsL*	*recA* derivative of TE13
TE0104	F$^-$	Amp^r-400		*pyrB thr leu recA rpsL*	Amp^r-400 mutant of TE01
SN07	Hfr	Amp^r-300			Amp^r-300 transductant of KL25

immunoelectrophoresis method of Laurell (23) was used. Agarose was dissolved in Veronal buffer at 100°C at a concentration of 1% (wt/vol). Rabbit antiserum against purified β-lactamase (24) was added at 45°C. Gels were prepared on glass plates (8 by 8 cm). Electrophoresis was at 8 V/cm. Cell extracts were prepared as follows. Bacteria were grown to an optical density of 100 Klett units in 100 ml of LB medium, centrifuged (23,000 × *g* for 15 min), and disrupted with a Branson sonifier B12. The sonic extracts were applied to wells in the agarose gel. The area under the precipitation line was taken as a relative value of the amount of β-lactamase. To verify the location of active β-lactamase molecules in the gels, an 8-ml solution of 1% agar (wt/vol), 1% starch (wt/vol), and 1 mM benzylpenicillin was applied to the agarose gels. Hydrolyzed benzylpenicillin was identified as a colorless area after application of an iodine-iodide solution.

Gel electrophoresis of proteins. The gel electrophoresis procedure was that of Laemmli and Favre (21). A slab gel apparatus with a 12.5-cm separating gel was used; 10% gels were run at 20 mA. Staining, destaining, and drying of gels were as described by Fairbanks et al. (14).

RESULTS

Isolation of β-lactamase-hyperproducing mutants. Spontaneous mutants of strain LA51 (*ampA1 ampC*$^+$) and its *recA* derivative, SN02, were selected on plates containing 50 μg of ampicillin per ml. The incidence of such mutants was the same in the two strains (about 10^{-8} per viable cell). The β-lactamase activities of 65 Amp^r-50 mutants are shown in Fig. 1. Most of the resistant mutants of both strains, LA51 and SN02 (*recA*), exhibited increased β-lactamase activity. The majority of the mutants showed activity that was approximately twice that of the respective parental *ampA1 ampC*$^+$ strain. In addition, mutants were found having β-lactamase activity three- to sixfold that of the parental strains.

The incidence of Amp^r-100 mutants was about 10^{-9} per viable cell. Twenty Amp^r-100 mutants were isolated, all exhibiting β-lactamase activity 7- to 10-fold that of parental strain LA51. Second-step mutants of Amp^r-100 were isolated with a three- to fourfold further increase in β-lactamase activity. They occurred at an incidence of 10^{-9} per viable cell.

Ampicillin resistance and β-lactamase activity were monitored in strains LA51 (Amp^r-10), TE01 (Amp^r-100), TE13 (Amp^r-100), and TE0104 (Amp^r-400) (Table 2). Ampicillin resistance correlated well with β-lactamase activity. The relative amount of β-lactamase was analyzed by immunoelectrophoresis with antibodies against purified β-lactamase (Table 2). The increased ampicillin resistance of strains TE01, TE13, and TE0104 was clearly caused by β-lactamase hyperproduction.

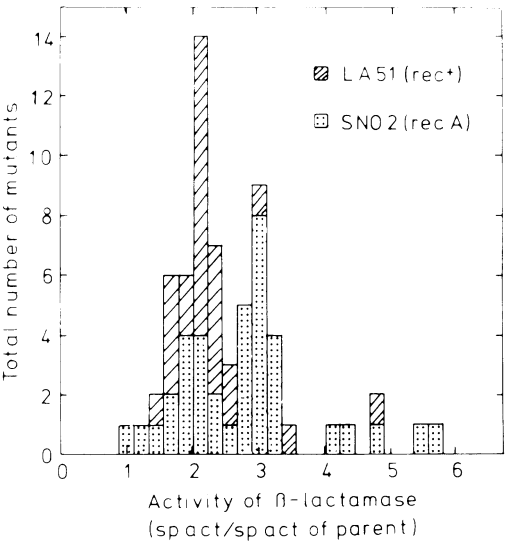

FIG. 1. *β-Lactamase activities of spontaneous mutants from strain LA51 and its recA derivative, SN02. The mutants were isolated on LA plates containing 50 μg of ampicillin per ml and were tested for β-lactamase activity as described in the text. The specific activities of strains LA51 and SN02 were 12 and 10 U/mg of protein, respectively. The activities of the mutants were normalized with respect to the respective parental strain. The data from each strain were added, and the total number is given. Thus, the 2+ column indicates 10 mutants of LA51 and 4 of SN02, for a total of 14.*

TABLE 2. *Resistance, activity, and amount of β-lactamase in highly ampicillin-resistant mutants*[a]

Strain	Ampicillin resistance (μg/ml)	Sp act of β-lactamase (U/mg of protein)	Relative amt of β-lactamase
LA51	10	12	1
TE01	100	115	10
TE13	100	100	10
TE0104	400	400	35

[a] Determined as described in the text.

A protein with the same molecular weight as the chromosomal β-lactamase was found to be one of the most abundant cellular proteins in strain TE0104 (Fig. 2). In strains TE01 and TE0104, an additional protein was found with a molecular weight of about 65,000. The nature of this protein is not known at present.

Transduction and conjugation analyses of β-lactamase-hyperproducing mutants. The *ampA1* allele and the structural gene for chromosomal β-lactamase are cotransducible with *purA* and *fdp* (13). The cotransduction frequencies have been estimated to be between 30 and 40% for *purA-ampAC* and 5% for *fdp-ampAC*. The *purA* and *fdp* loci were therefore

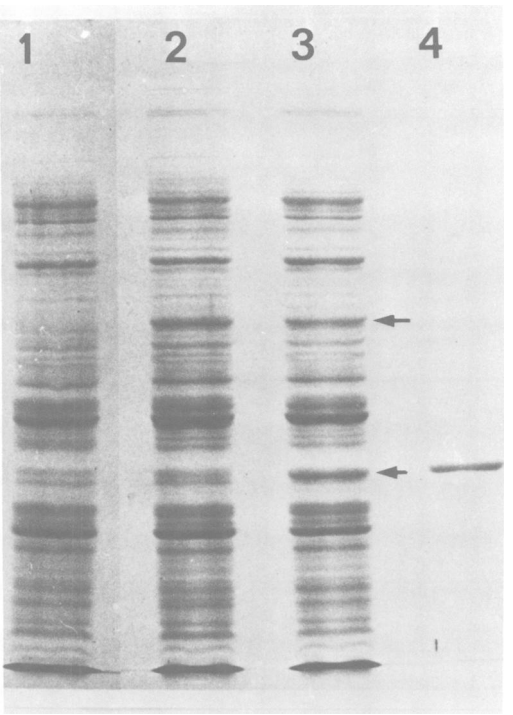

FIG. 2. *Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis of total sodium dodecyl sulfate-soluble protein from (1) LA51 (Amp^r-10) (2) TE01 (Amp^r-100), and (3) TE0104 (Amp^r-400). Strains were grown in minimal glucose medium and harvested at an absorbance at 450 nm of 0.5. The bacteria were boiled in 2% sodium dodecyl sulfate for 5 min, and 50 μg of protein was applied to each slot. Purified chromosomal β-lactamase (25 μg) was applied to slot 4. Electrophoresis in 10% polyacrylamide gel containing 0.1% sodium dodecyl sulfate was carried out at room temperature with a constant current of 20 mA/gel. The gels were stained in Coomassie brilliant blue. Arrows indicate positions in the gel of chromosomal β-lactamase and a second protein apparent in strains TE01 and TE0104.*

used in the genetic analyses of β-lactamase-hyperproducing mutants. A number of transduction experiments were performed with strain TE01 as the donor and strains PA256 (*ampA*$^+$ *ampC*$^+$ *purA*) and Q11 (*ampA*$^+$ *fdp*) as recipients. Ade$^+$ Fdp$^+$ or ampicillin-resistant (Amp^r) transductants were selected and scored for their ampicillin resistance. Several classes of ampicillin-resistant transductants were found. In Table 3 are given the cotransduction frequency between the selected marker and the ability to grow on different ampicillin concentrations. Single cells of PA256 derivatives carrying the *ampA1 ampC*$^+$ were resistant to 8 μg of ampicillin per ml, whereas the corresponding *ampA1 ampC*$^+$ derivative of strain Q11 tolerated 10 μg/ml. Thus, growth on 6 and 10 μg of ampicillin per ml was taken as evidence for Amp^r in derivatives of strains PA256 and Q11, respectively. The cotransduction estimate between *purA* and Amp^r-6 (growth on 6 μg of ampicillin per ml) was almost 30%. Gradual decreases in the cotransduction frequencies were found between *purA* and increasing Amp^r. Thus, very few Ade$^+$ transductants had received the full resistance of strain TE01. The estimates of cotransduction between *purA*$^+$ and Amp^r-100 was thus only 0.3%. Among 750 Fdp$^+$ transductants, 33 were Amp^r, i.e., 4.4% linked. Among these Amp^r transductants, only one had received the full resistance of strain TE01. Direct selection for Amp^r increased the frequency of clones showing an Amp^r-100 phenotype. Amp^r-100 clones occurred at a higher frequency among the isolated Amp^r transductants when the ampicillin concentration in the selective plate was increased. Thus, 80% of the PA256 transductants selected as Amp^r-50 were resistant to 100 μg of ampicillin per ml.

Strain TE13 was used as donor in similar transduction experiments (Table 3). Several classes of ampicillin-resistant transductants were found, as was the case with strain TE01. However, *purA* was apparently not cotransducible with Amp^r-100 in strain TE13. The maximal resistance transferred together with Ade$^+$ was to 30 μg of ampicillin per ml. Cotransduction between *purA* and Amp^r-6 was close to 40%. When directly selected for a specific Amp^r level, transductants with higher ampicillin resistance were found. This was especially evident when selecting for Amp^r-10 transductants of strain PA256. Of 100 such clones, 2 had inherited the full resistance of strain TE13. A summary of the transductions is given in Fig. 3, where we suggest that the Amp^r-100 phenotypes in both strains are due to multiple repetitions of a deoxyribonucleic acid (DNA) *amp* segment.

A number of Pur$^+$ Amp^r transductants were carefully analyzed with respect to their ampicillin resistance and β-lactamase activity (Fig. 4). Groups of transductants were found that differed from one another in the ampicillin resistance and the β-lactamase activity mediated by an *ampA1 ampC*$^+$-carrying PA256 strain. Ampicillin resistance correlated linearly to β-lactamase activity.

Segregation of several classes of ampicillin-resistant recombinants was also obtained in conjugations with either strain TE01 or TE13 as a recipient. The occurrence of intermediate-resistance classes was not dependent on the presence of the *ampC*$^+$ allele in the donor, since such classes were found in crosses where the β-lactamase-negative strain UM100 (*ampA ampC8*) was used as the donor.

TABLE 3. *Cotransduction estimates with strains TE01 and TE13 as donors*[a]

Donor	Recipient	Selected phenotype	No. of transductants	Cotransduction (%) between selected phenotype and different Amp[r] levels[b]							
				6	10	20	30	40	50	70	100
TE01	PA256	Ade+	300	27	25.3	11.7	6.3	3	2.7	0.6	0.3
TE01	PA256	Amp[r]-6	200	100	94.4	50.5	28.5	13	6	4.5	2.5
TE01	PA256	Amp[r]-10	200	100	100	88.5	71	43	32.5	20	13
TE01	PA256	Amp[r]-50	75	100	100	100	100	100	100	97.3	80
TE01	Q11	Fdp+	750	4.4	4.4	4.4	2.2	1.2	1.2	0.4	0.1
TE01	Q11	Amp[r]-10	200	100	100	83	47	28	27	16	5.5
TE01	Q11	Amp[r]-40	200	100	100	100	100	100	99.5	86.5	43
TE13	PA256	Ade+	300	39.0	37.3	5.6	2	<0.3	<0.3	<0.3	<0.3
TE13	PA256	Amp[r]-6	200	100	82.5	15.5	3	0.5	0.5	0.5	<0.5
TE13	PA256	Amp[r]-10	100	100	100	99	42	23	23	4	2
LA51	PA256	Pur+	200	37.1	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
LA51	Q11	Fdp+	200	5	5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5

[a] Transduction procedures were as described in the text. Ade+ and Fdp+ transductants were selected on minimal media, and Amp[r] transductants were selected on LA plates containing the indicated concentration of ampicillin. Clones were tested for ampicillin resistance. The percentage of selected transductants growing on indicated ampicillin concentrations is given. Phage P1*clr*100*cml* was used. Similar results were obtained with phage P1*c*.

[b] Micrograms of ampicillin per milliliter.

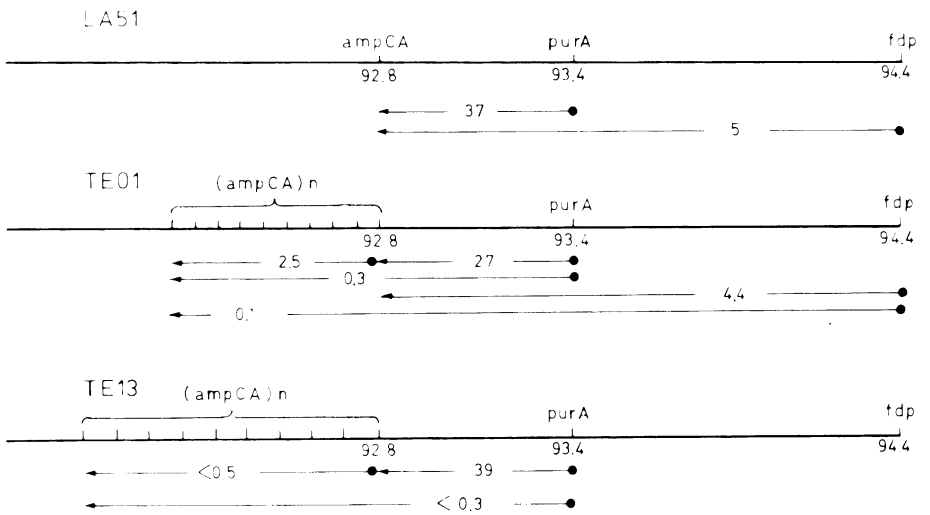

FIG. 3. *Genetic map of the ampAC region, with time indications and position of earlier known genes according to Bachmann et al. (1). The cotransduction frequencies given as percentages are from Table 3. The marks indicate assumed chromosomal repetitions. The distance between each amp repetition is not known.*

Strain TE0104, resistant to 400 μg of ampicillin per ml, was used as the donor in a transduction with strain PA256. Amp[r] transductants were isolated on LA plates containing 6 μg of ampicillin per ml and scored for their Amp[r] phenotype. Figure 5 gives the resistance of a number of Amp[r]-6 transductants. In this highly ampicillin-resistant mutant, resistance appeared to be built up of units, each giving a tolerance corresponding to that mediated by the combination *ampA1 ampC*[+]. The highest possible resistance transduced into strain PA256 was about 200 μg of ampicillin per ml.

An Amp[r]-300 transductant of Hfr KL25, strain SN07, was obtained in a P1 cross with strain TE0104 (Amp[r]-400) as the donor. An interrupted-mating experiment was performed with SN07 as the donor and LA5 (*ampA*[+] *ampC*[+]) as the recipient. Selection was made for Amp[r]-10, Amp[r]-200, and Ura[+] clones (Fig. 6A). Amp[r]-10 recombinants appeared about 5 min before Ura[+] clones. The number of Amp[r]-200 clones was low; nevertheless, the entrance of Amp[r]-200 occurred before that of Ura[+]. The distance between the entrance of Amp[r]-10 and Amp[r]-200 was tentatively found to be 2 to 3 min. Recombinants selected as Amp[r]-200 were tested for their Ura phenotype. It was found that the fre-

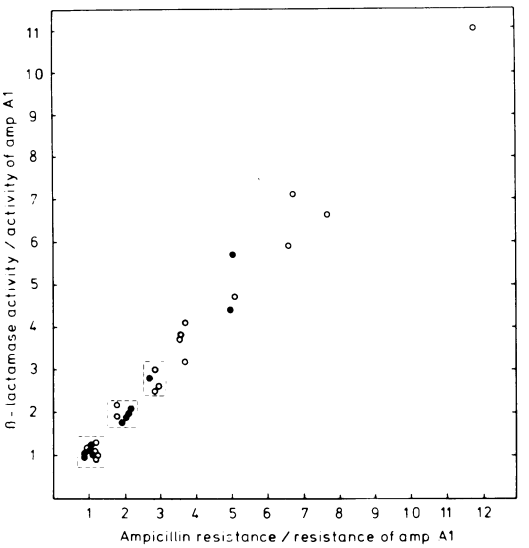

FIG. 4. *β-Lactamase activity of 31 Pur⁺ Amp^r transductants from crosses P1v (TE01) × PA256 and P1v (TE13) × PA256 was determined as described in the text. Resistance to ampicillin for single cells was determined as described previously (9). The specific β-lactamase activity of an ampA1-carrying PA256 was 10 U/mg of protein, and the resistance was 8 μg of ampicillin per ml. The specific activity and ampicillin resistance of each transductant were normalized with respect to an ampA1-carrying PA256 strain. Symbols: ○, transductants from cross P1v (TE01) × PA256; ●, transductants from cross P1v (TE13) × PA256.*

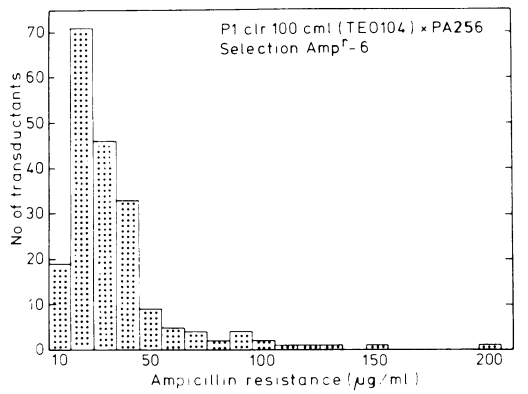

FIG. 5. *Ampicillin resistance of transductants obtained in cross P1clr100cml × PA256, selecting for Amp^r-6. Transductants were tested for their resistance by replica plating (8). The numbers of transductants in different resistance groups are given.*

quency of Ura⁺ clones was low at times early after the entrance of Amp^r-200, showing that the entire ampicillin resistance of strain SN07 was transferred before *pyrB*. Among the Amp^r-10 recombinants, those showing higher resistance levels were also Ura⁺ at much higher frequency than those showing a low ampicillin resistance. Among recombinants selected as Ura⁺, 30% were wild type, whereas 57% exhibited a resistance to concentrations ranging between 10 and 150 μg/ml. The remaining 13% were Amp^r-200.

In a control experiment, strain SN06 (*ampA1 ampC⁺*) was crossed with strain LA5, and Amp^r-10 and Ura⁺ recombinants were selected. Amp^r-10 recombinants appeared after 22 min of conjugation, and Ura⁺ recombinants appeared 2 min later (Fig. 6B).

Genetic stability. The β-lactamase-hyperproducing strains were not stable. To isolate segregants, strains TE01 (Amp^r-100) and TE13 (Amp^r-100), their *recA* derivatives (SN04 and SN05), and strain TE0104 (Amp^r-400) were

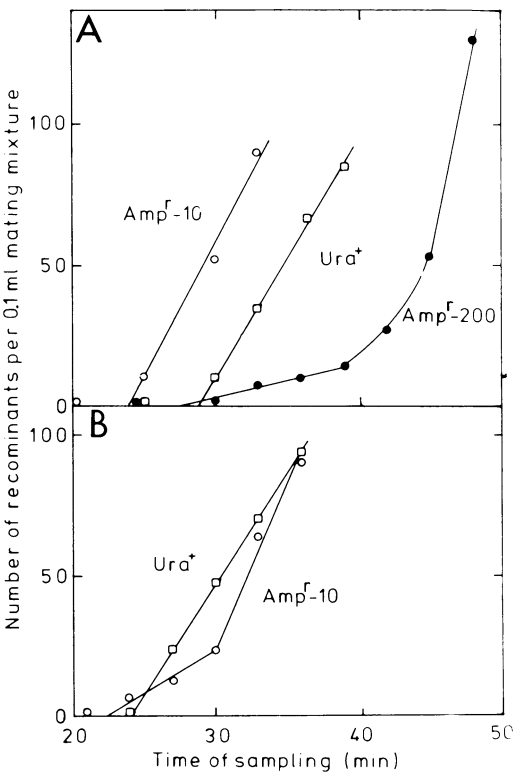

FIG. 6. *(A) Interrupted-mating experiment in which strain SN07 (Amp^r-300) was crossed with strain LA5 (ampA⁺ ampC⁺). Selection for ampicillin resistance was done on LA plates containing 10 (○) or 200 μg (●) of ampicillin per ml. Ura⁺ recombinants were obtained on minimal medium plates (□). (B) Interrupted-mating experiment in which strain SN06 (ampA1 ampC⁺) was crossed with strain LA5. Selection was done for Ura⁺ and for Amp^r-10 recombinants. Counterselection of the donor was obtained by streptomycin (100 μg/ml) in both crosses.*

grown in LB medium for 30 generations. Two hundred clones from each strain were tested for their ampicillin resistance (Table 4). A number of TE01 and TE13 segregants were found with resistance to ampicillin concentrations ranging between 10 and 90 μg/ml. Segregation was more pronounced in strain TE13, which apparently has larger *amp* repetitions. In the *recA* strain SN04, no segregants were found, whereas at least two SN05 clones clearly showed a lower resistance, suggesting stabilization of the genotype in a *recA* background. From the Amp^r-400 mutant TE0104, a large number of segregant classes were found. This segregation pattern was an additional evidence that this strain is built up of *ampA1 ampC*$^+$ repetitions.

Ampicillin-resistant mutants of wild-type strains. The genetic data given above suggested that β-lactamase-hyperproducing mutants contained repetitions of chromosomal *amp* genes. Since the parental strains in all cases contained the *ampA1* allele, it could be argued that this allele was a prerequisite for the formation of repetitions. If this is not the case, it should be possible to isolate moderately ampicillin-resistant mutants of wild-type strains similar to our β-lactamase-hyperproducing mutants. The original *ampA1* strain was isolated from strain G11 (13). This strain was therefore used to isolate spontaneous mutants resistant to ampicillin concentrations ranging between 6 and 20 μg/ml. Mutants resistant to 8 or more μg of ampicillin per ml showed increased β-lactamase activity. Transduction experiments were made with seven such mutants as donors and with the wild-type PA256 as a recipient. With the control strain G11a1 (*ampA1 ampC*$^+$) as the donor, 25% of the Ade$^+$ transductants were *ampA1*, i.e., resistant to 8 μg of ampicillin per ml. The new moderately resistant mutants isolated showed completely different genetics (Table 5). Most Ade$^+$ transductants showed ampicillin resistance indistinguishable from that of the recipient. In addition, transductants were found that exhibited slightly higher tolerance towards ampicillin and that showed β-lactamase activity approximately two, three, or four times that of strain PA256. Thus, these mutants clearly were not mutated in *ampA*. Instead, they appeared to contain repetitions of the wild-type *ampA*$^+$ and *ampC*$^+$ alleles.

β-Lactamase activity and ampicillin resistance of merodiploid β-lactamase-hyperproducing strains. The genetic event leading to β-lactamase hyperproduction regardless of episomal or chromosomal location was domi-

TABLE 4. *Ampicillin resistance of segregants*[a]

Strain	No. of clones tested	No. of clones in different Amp^r resistant classes														
		1[b]	10	20	50	70	80	90	100	150	200	250	300	350	400	500
TE01 (Amp^r-100)	200	0	0	1	5	5	0	1	193	0	0	0	0	0	0	0
SN04 (Amp^r-100)	200	0	0	0	0	0	0	0	200	0	0	0	0	0	0	0
TE13 (Amp^r-100)	200	0	1	6	1	3	6	10	173	0	0	0	0	0	0	0
SN05 (Amp^r-90)	200	0	0	0	0	2	105	93	0	0	0	0	0	0	0	0
TE0104 (Amp^r-400)	200	0	2	8	5	3	0	4	10	41	25	39	25	25	11	0

[a] Strains TE01 (Amp^r-100) and TE13 (Amp^r-100), their *recA* derivatives, SN04 and SN05, and TE0104 (Amp^r-400) were grown in LB medium for about 30 generations and then plated on LA without ampicillin. A number of clones from each strain were tested for ampicillin resistance by replica plating.

[b] Micrograms of ampicillin per milliliter.

TABLE 5. *Ampicillin resistance of Ade$^+$ transductants in crosses between moderately ampicillin-resistant mutants and strain PA256*[a]

Donor	Ampicillin resistance (μg/ml)	Sp act of β-lactamase (U/mg of protein)	No. of Ade$^+$ transductants tested	Ade$^+$ transductants in different resistance classes (%)					
				1[b]	2	3	4	5	8
G11a1	20	14	200	75	0	0	0	0	25
G11	2	1	200	100	0	0	0	0	0
G11m1	20	10	100	98	1	1	0	0	0
G11m2	12	7	100	92	6	2	0	0	0
G11m3	10	7	100	98	2	0	0	0	0
G11m4	8	5	100	96	4	0	0	0	0
G11m5	14	10	100	98	2	0	0	0	0
G11m6	20	14	200	87	10	2	1	0	0
G11m7	20	14	200	92	8	0	0	0	0

[a] Strains G11m1 through G11m7 are G11 mutants moderately resistant to ampicillin. The experimental conditions were as described in footnote *a* of Table 3. The percentage of Ade$^+$ transductants in different resistance classes is given. Strain PA256 is more ampicillin sensitive than strain G11 (1 and 2 μg of ampicillin per ml, respectively). Wild-type repetitions must therefore express a lower resistance in strain PA256 than in strain G11.

[b] Micrograms of ampicillin per milliliter.

nant over *ampA*$^+$ as well as over *ampA1* (Table 6). Moreover, the resistance and β-lactamase activity of each merodiploid strain was approximately the sum of those of the respective haploid strains. The F′ factor 1182 (*ampA1 ampC12*) codes for a heat-labile chromosomal β-lactamase (31). Cell extracts of strain SN04/F′1182 grown at 28°C contained mainly the temperature-resistant enzyme coded for by the chromosome (Table 6). Thus, the expression of β-lactamase hyperproduction was not affected by the presence of tested *ampA* and *ampC* alleles in *trans* and did not act in *trans* on these alleles.

DISCUSSION

The *ampA1* mutation close to the structural gene *ampC* leads to a 10-fold increase in ampicillin resistance and β-lactamase production (24). Since *ampA1* acts in *cis* but not in *trans*, *ampA* is thought to be the operator-promoter region for *ampC* (31).

The increase in β-lactamase activity of the highly ampicillin-resistant mutants studied here could be explained by one of the following hypotheses: the mutants (i) harbor repeated sequences of *ampA1 ampC*$^+$; (ii) carry repetitions of *ampA1* but not of *ampC*$^+$, or repetitions of *ampC*$^+$ but not of *ampA1*; (iii) contain mutations in a regulatory gene leading to an increase in the efficiency of *ampC* transcription; (iv) produce a β-lactamase with an increased specific activity against ampicillin.

The last possibility is unlikely, since the very-ampicillin-resistant strains, TE01, TE13, and TE0104, produce considerably more β-lactamase than an *ampA1 ampC*$^+$ strain as measured either by specific antibodies against purified β-lactamase, by protein separation on sodium dodecyl sulfate slab gels, or by the actual amount of enzyme obtained after purification (data not shown). In the Amp^r-400 mutant TE0104, a protein with the same molecular weight as chromosomal β-lactamase is one of the major proteins in the cell.

Genetic characterization of the β-lactamase-hyperproducing mutants TE01, TE13, and TE0104 strongly suggests that repetitions of a DNA segment carrying the *ampA1* and *ampC*$^+$ alleles cause increased β-lactamase production. From these strains it was possible to obtain several classes of transductants, each differing in ampicillin resistance and β-lactamase activity from those mediated by an *ampA1 ampC*$^+$ strain. These repetitions must be chromosomal, because they are cotransducible with the chromosomal genes *purA* and *fdp* and because they may be localized by conjugation.

Moderately ampicillin-resistant mutants could be isolated from wild-type strains, which were not mutant in *ampA*. The transduction data were compatible with the idea that resistance was brought about by repetitions of the wild-type (*ampA*$^+$ *ampC*$^+$) alleles. Thus, the *ampA1* mutation per se is not a prerequisite for gene repetitions.

The *ampA1* mutation is 30 to 40% cotransducible with *purA* (13). In strains TE01 and TE13, *purA* and Amp^r-6 (growth on 6 μg of ampicillin per ml) were 27 and 39% cotransduced, respectively. It is therefore likely that the distance from *purA* to the closest *ampA1* or *ampC*$^+$ allele is unaffected in the β-lactamase-hyperproducing mutants, i.e., about 0.6 min (1). In strain TE01, the Amp^r-100 phenotype showed 0.3% cotransduction with *purA*, whereas no cotransduction was found between Amp^r-100 and *purA* in strain TE13. This suggested a larger map expansion in the latter strain. When the different cotrans-

TABLE 6. *Dominance relationship of β-lactamase hyperproducers*[a]

Strain	Chromosome			Episome			Sp act of β-lactamase (U/mg of protein)	Ampicillin resistance (μg/ml)
	Genotype		Phenotype	Genotype		Phenotype		
	ampA	*ampC*		*ampA*	*ampC*			
SN04			Amp^r-100				119	100
SN04/F′118			Amp^r-100	+	+		125	100
SN04/F′1181			Amp^r-100	1	+		131	110
SN04/F′1183			Amp^r-100			Amp^r-100	191	200
SN01/F′1183	+	+				Amp^r-100	120	110
SN02/F′1183	1	+				Amp^r-100	143	120
SN04/F′1182			Amp^r-100	1	12		131 (28°C)	NT
							130 (44°C)	NT

[a] Merodiploid strains were grown in Casamino Acids medium. For strain SN04, uracil (25 μg/ml) was included in the medium. Enzyme extracts were prepared and assayed for β-lactamase activity as described in the text. Enzyme extract of strain SN04/F′1182 was prepared from cells grown at 28°C. The extract was incubated at 28 or 44°C for 1 h. Resistance for single cells was determined as described in the text. NT, Not tested.

duction data were converted to map distance by the formula of Wu (37), the segment covered by *amp* repetitions was in the order of 1 min in strain TE01 and 1.5 min in strain TE13. Both strains showed about 10 times the resistance and β-lactamase activity of strain LA51 (*ampA1 ampC*$^+$). It has previously been found by using merodiploid strains that the *ampA1* and *ampC*$^+$ alleles show an absolute gene dosage effect (31). Therefore, the number of gene repetitions in the two mutants must be close to 10. Each repetition would thus be about 0.1 min in strain TE01 and about 0.15 min in strain TE13, corresponding roughly to 5,000, and 8,000 base pairs, respectively. Since the β-lactamase itself, with a molecular weight of 31,400 (24), can only be coded for by approximately 800 base pairs, each repetition must consist of considerably more DNA than is comprised by the *ampA1* and *ampC* alleles. Strain TE0104 is a spontaneous Amp^r-400 mutant of TE01 and shows a β-lactamase activity about 40 times that of an *ampA1 ampC*$^+$ strain. If this is correlated with gene frequency, this strain must harbor about 40 *amp* repetitions. The maximum transducible segment for phage P1 has been determined to be 2.3 min (1). Thus, phage P1 can, at most, transduce 23 repetitions with a length of 0.1 min. This fits with the finding that it was possible to transduce an Amp^r-300 phenotype, but not a higher resistance level, into a sensitive strain.

In the interrupted-mating experiment with the Amp^r-300 transductant strain SN07 as the donor, Amp^r-10 recombinants appeared about 5 min before Ura$^+$ clones. This should be compared with the distance of ca. 2 min between *ampA1* and *pyrB* in an Amp^r-10 strain. Moreover, Amp^r-200 clones appeared at least 2 min after entrance of Amp^r-10. Few recombinants were Amp^r-200; they were considerably less abundant than Ura$^+$. However, any crossover within the region *ampAC* repetition would lead to decreased ampicillin resistance. This was clearly evident when Ura$^+$ recombinants were tested for their ampicillin resistance, where only 13% were Amp^r-200. Among the Ura$^+$ recombinants, 30% showed a wild-type resistance. They must have been formed by a recombinational event between the last injected *ampAC* repetitions and *pyrB*. Ura$^+$ recombinants with intermediate resistance amounted to 57%, suggesting free recombination between the distal *ampAC* genes. With strain SN06 (*ampA1 ampC*$^+$), the *pyrB* marker was transferred after 24 min of conjugation, whereas the entrance time for the same gene with strain SN07 (Amp^r-300) was 28 to 29 min. Taken together, strain SN07 appears to contain a DNA addition of 2 to 3 min comprised of about 30 *ampAC* repetitions. It should be noted, however, that other effects such as inhibition of recombination due to homology and nonhomology junctions might affect apparent marker distances. Apparent gene duplications and gene repetitions of *amp* genes occurred spontaneously in *recA* as well as in Rec$^+$ strains with frequencies that were of the same order of magnitude. The independence of gene duplications and repetitions from the *recA* function suggest a mechanism involving "illegitimate" recombination (15), as has been proposed for gene duplications in the *arg* region of the chromosome (2). It has been suggested that generation of tandem duplications may involve uneven recombination between the two replicating arms close to a replication fork (2, 23). Whether or not gene repetitions of *ampAC* occur at the replication fork is not known. However, preliminary data have shown that nitrosoguanidine increases the incidence of β-lactamase-hyperproducing mutants. Since this mutagen acts primarily at the replication fork (10), gene repetitions may be formed predominantly from newly replicated DNA.

The incidence of mutants with 10 *amp* repetitions was only 10-fold lower than the incidence of mutants with two to three apparent *amp* repetitions, suggesting that gene repetitions are not caused by independent gene duplications. The mutation experiments showed that roughly 10 *amp* repetitions was the highest number obtained in one mutational event. Since a second selection resulted in mutants with higher apparent *amp* copy number, the gene expansion as such does not set the limit for the number of repetitions. These second-step mutants might therefore result from repetitions of the *amp* repetition region of the parental strain.

The β-lactamase-hyperproducing mutants were not stable. It was possible to isolate segregants from strains TE01, TE13, and TE0104. As expected, these segregants fell into different resistance classes, suggesting loss of different numbers of *ampAC* repetitions. The *recA* allele apparently stabilized the repetitions. Such a stabilization with *recA* has also been observed for tandem duplications (2).

Inverted DNA repeat sequences (IS sequencies) have been demonstrated in a number of plasmids of the F and R types as well as on the *E. coli* chromosome (12, 18, 19, 34). Such sequences have been implicated in *recA*-independent recombination events (3, 20). It is possible, but has not been shown, that DNA repeats that bracket resistance genes are also involved in the formation of multiple copies of R-plasmid resistance determinants (32, 33). We have not yet been able to transpose chromosomal *amp* genes to plasmids, which suggests that the DNA-*amp*

repetitions are not similar in nature to the transposable resistance elements on R-plasmids. The ultimate answer to the organization of *amp* repetitions must await until recently isolated λdAmp^r-100 transducing phages are fully analyzed.

In conclusion, development of chromosomal resistance to β-lactam antibiotics in *E. coli* K-12 most readily occurs by increasing the production of wild-type chromosomal β-lactamase. This may be achieved either by promoter-operator-like mutations in *ampA* or by apparent repetitions of the β-lactamase genes. A combination of these mutational events may lead to very-ampicillin-resistant strains. The strong and simple selection procedure for mutants with increased chromosomal β-lactamase is a feature that makes the *amp* genes suitable for the study of gene duplications and gene repetitions. Hopefully it will also yield information to the role these kinds of mutations may have played in evolution and may play in the evolution of plasmid-linked resistance determinants. It should be noted that an R-plasmid has recently been shown to code for a β-lactamase indistinguishable from the chromosomal β-lactamase of *E. coli* (5).

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