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Resistance of *Escherichia coli* to Penicillins: Fine-Structure Mapping and Dominance of Chromosomal Beta-Lactamase Mutations

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Seven *Escherichia coli* K-12 mutants with a lowered chromosomal β-lactamase activity were analyzed genetically. The β-lactamase-negative mutants isolated from *ampA1*-carrying strains (resistant to 10 μg of ampicillin per ml) all carried genetic lesions very close to the *ampA1* mutation, which was still present. In an earlier report, two of the mutations mediating a β-lactamase-negative phenotype (L. G. Burman, T. Park, E. B. Lindström, and H. G. Boman, J. Bacteriol. 116:123-130, 1973) were shown to have occurred in the structural gene for β-lactamase, designated *ampC*. It is suggested that all β-lactamase-negative mutants studied here were altered in *ampC*. The relative order of *ampC* mutations was (*ampC1*, *ampC8*)-*ampC9*-(*ampC12*, *ampC14*)-*ampC11*, and the gene order was found to be *ampC-ampA-purA*. The *ampA1* allele was dominant over its wild-type allele but acted only *cis* and not *trans*, suggesting that *ampA* is the promoter or operator region for *ampC*. A gene dosage effect was found for strains homozygous for *ampA*$^+$ *ampC*$^+$ or *ampA1* *ampC*$^+$. Heterozygotes carrying the *ampC8* allele on the chromosome showed an apparent derepression of the episomal *ampC* allele, suggesting a role for β-lactamase in its own regulation.

Wild-type *Escherichia coli* K-12 produces a constitutively small amount of a chromosomally mediated β-lactamase. This enzyme activity does not contribute to the penicillin resistance of wild-type strains, which is on the order of 1 to 2 μg of ampicillin per ml. It has therefore been suggested that the chromosomal β-lactamase fulfills another function in the cell (3, 5). *E. coli* K-12 mutants resistant to 10 to 20 μg of ampicillin per ml can be isolated at an incidence of 10^{-9} per viable cell. One gene involved in this alteration was designated *ampA* and was located at 93 min on the revised chromosomal map of *E. coli* (1, 8, 9). Strains carrying the *ampA1* allele contain about 10 times the amount of β-lactamase found in wild-type strains (10). β-Lactamase purified from strains carrying the wild-type or *ampA1* allele showed no differences in enzymatic properties (10). These data therefore suggested that *ampA* was a regulatory locus for chromosomal β-lactamase. Mutants defective in production of β-lactamase have been isolated 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* (5). By transduction, *ampC* was located to the *ampA-purA* region of the chromosome (4, 5).

The genetic and regulatory relationships between *ampA* and *ampC* was further analyzed here by the use of β-lactamase-negative mutants and of strains diploid for the *ampA-ampC* region.

MATERIALS AND METHODS

Organisms. The *E. coli* K-12 strains used are listed in Table 1. Strains D21, LA51, and R12 are transductants carrying the *ampA1* allele. R12 is a derivative of Hfr Reeves 1 and injects its genes in the order: origin-*metB-ampA1-thr-lac*.

The isolation of β-lactamase-negative mutants from the *ampA1*-carrying strains D21 and R12 was recently described (5). Here we make use of three β-lactamase-negative female strains studied previously, TP1 (*ampC1*), H1t8 (*ampC3*) and H1t14 (*ampC12*) (5), as well as of four mutants isolated from Hfr R12 by the same procedure, UM100 (*ampC8*), UM101 (*ampC9*), UM102 (*ampC11*), and UM103 (*ampC14*). Strain H1t14 (*ampC12*) contains a heat-labile β-lactamase (5). The other mutants exhibit a very low β-lactamase activity irrespective of growth temperature (≤1% of the parental activity).

The strains used for the dominance studies were constructed as follows. The *ampC8* allele was intro-

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

Strain	Source and/or reference	Mating type	Genotype		Other relevant markers
			ampA	*ampC*	
G11	G. G. Stent	Hfr	+	+	*metB, ilv*
R12	5	Hfr	1	+	*metB*
KL16-99	K. B. Low; 12	Hfr	+	+	*recA*
KL132/KLF18	K. B. Low; 12	F′	+	+	*pyrB, thy, leu, thr, his, rpsL*, F′118
D11	H. G. Boman	F⁻	+	+	*proA, trp, his, rpsL*
D21	H. G. Boman	F⁻	1	+	*proA, trp, his, rpsL*
Q11	D. G. Fraenkel; see also ref. 8	F⁻	+	+	*fdp*
Q111	Streptomycin-resistant mutant of Q11; this paper	F⁻	+	+	*fdp, rpsL*
PA256	R. Lavallé; see also ref. 8	F⁻	+	+	*pro, his, argF, purA, rpsL*
PA2561	Nalidixic acid-resistant mutant of PA256; this paper	F⁻	+	+	*pro, his, argF, purA, rpsL, nal*
PA2004	R. Lavallé	F⁻	+	+	*thr, leu, his, pyrB, rpsL*
LA51	*ampA1* transductant of PA2004; this paper	F⁻	1	+	*thr, leu, his, pyrB, rpsL*
LA511	*ampC8* recombinant from cross UM100 × LA51	F⁻	1	8	*pyrB, his, rpsL*
SN01	*recA* derivative of PA2004; this paper	F⁻	+	+	*thr, leu, pyrB, rpsL, recA*
SN02	*recA* derivative of LA51; this paper	F⁻	1	+	*thr, leu, pyrB, rpsL, recA*
SN03	*recA* derivative of LA511; this paper	F⁻	1	8	*pyrB, rpsL, recA*

duced into strain LA51 (*ampA1 ampC*⁺) by conjugation. *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 (6). Derivatives of the F′118 (*ampA*⁺ *ampC*⁺ *purA*⁺ *pyrB*⁺) were used in the dominance studies.

To isolate an episome carrying *ampA1 ampC*⁺, F′118 was introduced into strain D21 (*ampA1 ampC*⁺). Merodiploid clones were isolated on LA plates (see below) containing 40 μg of ampicillin per ml, i.e., twice the tolerance level of strain D21. Resistant clones appeared at a frequency of about 10^{-3} per donor cell. Segregants with the same resistance as strain D21 could be isolated. Twenty-five resistant merodiploid clones were mated with the Amp^s strain SN01 (*ampA*⁺ *ampC*⁺ *pyrB recA*), and Ura⁺ and Amp^r-10 (tolerance to 10 μg of D-ampicillin per ml) recombinants were selected. All 25 merodiploid D21 clones transferred ampicillin resistance, together with the Ura⁺ character, to strain SN01 (*ampA*⁺ *ampC*⁺). The F′ factor of one such heterozygous strain was denoted F′1181 (*ampA1 ampC*⁺) and was introduced into strains carrying different alleles of *ampA* and *ampC*.

F′1182 (*ampA1 ampC12*), coding for a heat-labile β-lactamase, was constructed as follows. F′1181 (*ampA1 ampC*⁺) was introduced into strain H1t14 (*ampA1 ampC12*) by selection for Amp^r-10 clones. One such clone was grown in LB medium (see below) and crossed with strain SN01 (*ampA*⁺ *ampC*⁺ *recA*). Merodiploids were selected as Ura⁺ SN01 clones. Of 100 diploid clones, 9 were found to be Amp^s at 42°C. The assumed episomal genotype of these diploids was *ampA1 ampC12*. To show that *ampA1* was still present on the episome, one merodiploid clone containing F′1182 was crossed with strain PA2004 (*ampA*⁺ *ampC*⁺). In this cross, Amp^r recombinants were obtained at a frequency of about 10^{-5} per donor cell.

The presence of F′118 (*ampA*⁺ *ampC*⁺), F′1181 (*ampA1 ampC*⁺), and F′1182 (*ampA1 ampC12*) was tested by a mating on plates (with PA256 as the recipient strain) and selection for Ade⁺ progeny. When merodiploids of strain SN03 were tested, a derivative of PA256, strain PA2561, which is chromosomally resistant to nalidixic acid, was used as the recipient to allow counterselection of the donor by nalidixic acid.

Media and growth conditions. The minimal medium used was medium E (17) supplemented with 0.2% glucose, 1 μg of thiamine per ml, 25 μg of the L-epimers of the required amino acids per ml, and, when necessary, 25 μg of uracil per ml. The Casamino Acids medium contained basal minimal medium, 0.2% casein hydrolysate, 0.2% glucose, and 25 μg of uracil per ml, when required. The complete medium was LB, as described by Bertani (2), supplemented with medium E, thiamine (1 μg/ml), and 0.2% glucose. It was solidified with 1.5% agar (LA plates). 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 Merck, Darmstadt, Germany.

Determination of ampicillin resistance. Ampicillin resistance was carefully determined for single cells as previously described (16).

Determination of β-lactamase activity. Cells growing exponentially in Casamino Acids medium were harvested and suspended in 0.05 M phosphate buffer, pH 7.4. Cells were lysed by the addition of

ethylenediamine tetraacetic acid (10^{-3} M) and lysozyme (100 μg/ml). β-Lactamase activity in the lysate was assayed by the automated micro-iodometric method, with benzylpenicillin (1,000 μg/ml) as the substrate (11). 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. (13), with bovine serum albumin as the standard.

Mating procedures. In the fine-structure mapping of β-lactamase-negative mutants, conjugation experiments resulted in a limited number of recombinants. The yields were therefore optimized as follows. The donor strain was cultivated at 37°C without aeration in a tube with LB, and the F^- strain was incubated in LB with strong aeration. For mating, 0.5 ml of the donor culture and 4.5 ml of the recipient culture (both 4×10^8 cells per ml) were mixed in a 100-ml flask. The mating mixture was incubated at 37°C for 2 h without shaking. Conjugation experiments with F′ strains as donors were performed as previously described (16).

RESULTS

Fine-structure mapping of β-lactamase-negative mutants. Ampicillin-resistant (Amp^r) recombinants of the genotype *ampA1 ampC*$^+$ should be obtainable by conjugation experiments by using two ampicillin-sensitive strains, one being a mutant Hfr (*ampA1 ampC*) and one being wild-type F^- (*ampA*$^+$ *ampC*$^+$). Such recombinants were found in crosses with the four β-lactamase-negative donors, UM100, UM101, UM102, and UM103. Thus, these four mutants are not *ampA*$^+$ revertants but carry mutations in a locus very close to the *ampA1* allele, probably in *ampC*. By the use of F^- strains carrying markers close to *ampA1* (*purA* and *fdp*), the relative order between *ampC* and *ampA* was investigated. Amp^r recombinants from such conjugation experiments were tested with respect to the *purA* and *fdp* alleles. The fact that the majority of the Amp^r recombinants were not adenine requiring (Ade^+) in crosses 2 to 5 (Table 2) indicated the gene order *ampC-ampA1-purA* in all four mutants.

When the four β-lactamase-negative donors (UM100 to UM103) were crossed with a wild-type recipient, reproducible differences between the donors in the number of Amp^r recombinants were obtained. All four Hfr mutants were therefore crossed to strains PA256 and PA2004 in parallel mating experiments. In each conjugation, the yield of Amp^r recombinants was related to the yield of another class of selected recombinants (Ade^+ or Ura^+). These mating experiments are summarized in Table 2 (crosses 2 to 9). The relative number of Amp^r recombinants in each cross was between 0.10 and 0.49%. Parallel results were obtained when strains PA256 and PA2004 were used as recipients. Assuming that recombination frequency is an approximate reflection of map distance, the following order of mutations was obtained: *ampC8-ampC9-ampC14-ampC11-ampA1-purA*.

Purified β-lactamase from strains carrying

TABLE 2. *Conjugation experiments with β-lactamase-negative mutants as donors and wild-type strains as recipients*

Cross	Donor[a]	Recipient	Relative frequency[b] of Amp^r recombinants (%)	Frequency of unselected markers among Amp^r recombinants[c] (%)	
				Ade^+	Fdp^+
1	UM100 (*ampA1 ampC8*)	Q111			45
2	UM100 (*ampA1 ampC8*)	PA256	0.49	76	
3	UM101 (*ampA1 ampC9*)	PA256	0.46	71	
4	UM102 (*ampA1 ampC11*)	PA256	0.17	75	
5	UM103 (*ampA1 ampC14*)	PA256	0.20	70	
6	UM100 (*ampA1 ampC8*)	PA2004	0.33		
7	UM101 (*ampA1 ampC9*)	PA2004	0.25		
8	UM102 (*ampA1 ampC11*)	PA2004	0.10		
9	UM103 (*ampA1 ampC14*)	PA2004	0.12		

[a] The donor strains inject their genes in the order: origin-*metB-ampA1-purA-fdp-pyrB*.

[b] The frequency of Amp^r recombinants is given as a percentage of the recombinants for Ade^+ (crosses 2 to 5) or Ura^+ (crosses 6 to 9). One hundred to 1,000 recombinants of each type were counted. *ampA1 ampC*$^+$ recombinants of strain PA256 (*ampA*$^+$ *ampC*$^+$) show unusually low resistance (5). Amp^r recombinants were therefore selected on LA plates containing 6 μg of ampicillin per ml when PA256 was used and 10 μg of ampicillin per ml when PA2004 (*ampA*$^+$ *ampC*$^+$) was used as the recipient. Ade^+, Ura^+, and Fdp^+ recombinants were selected on plates containing minimal medium.

[c] 100 Amp^r recombinants were tested in each cross.

the combination *ampA1 ampC3* or *ampA1 ampC12* show qualitative differences compared with the wild-type enzyme (5). It was therefore of particular interest to locate their *ampC* mutations relative to the β-lactamase mutations *ampC8*, *ampC9*, *ampC11*, and *ampC14* studied above. Amp^r recombinants were selected in conjugation experiments by using UM100, UM101, UM102, and UM103 as donors carrying *ampC8*, *ampC9*, *ampC11*, and *ampC14* and as recipients carrying *ampC3* and *ampC12*. When strain H1t14 (*ampA1 ampC12*) was used as recipient, the yield of Amp^r recombinants was between 0.006 and 0.11% (Table 3, crosses 10 to 13). An exceedingly low relative frequency (0.006%) was found in the cross with strain UM103 (*ampA1 ampC14*). This indicated that the *ampC12* and *ampC14* mutations are very close. The *ampC3* mutation of strain H1t8 was located in the same cluster, although it showed no strong linkage to any particular β-lactamase mutation in the donor strains (Table 3, crosses 14 to 17). A recipient strain (TP1) carrying *ampA1 ampC1* was also crossed with the four donor strains, UM100, UM101, UM102, and UM103 (Table 3, crosses 18 to 21). The *ampC1* mutation appeared to be very close to *ampC8* (Table 3, cross 18). In all of these apparently intragenic crosses (Table 3, crosses 10 to 21), the recombination frequencies obtained mirrored the order of *ampC* mutations deduced from crosses 2 to 9 in Table 2. The three female strains, H1t14 (*ampC12*), H1t8 (*ampC3*), and TP1 (*ampC3*) were also crossed to wild-type donor strains, and Amp^r recombinants were selected (Table 3, crosses 22 to 24). Such recombinants were easily obtained, showing that the female β-lactamase-negative strains also contained the *ampA1* allele. A tentative genetic map based on results given in Tables 2 and 3 is shown in Fig. 1.

β-Lactamase activity and ampicillin resistance of merodiploid strains. The dominance characteristics of the *ampA*$^+$ and *ampA1* alleles were studied in merodiploids by introducing the episomes F'118 (*ampA*$^+$ *ampC*$^+$), F'1181 (*ampA1 ampC*$^+$), and F'1182 (*ampA1 ampC12*) into *recA* derivatives of strain PA2004. Due to its *ampC12* allele, F'1182 mediates a heat-labile β-lactamase. The resulting merodiploids were tested for β-lactamase activity and ampicillin resistance (Table 4). *ampA1*

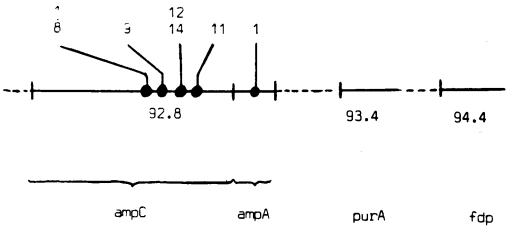

FIG. 1. *Tentative map of the β-lactamase region of the E. coli K-12 chromosome, including the ampC and ampA mutational sites used. The numbers above the line refer to mutant allele numbers. Time indications are according to Bachmann et al. (1).*

TABLE 3. *Conjugation experiments between β-lactamase-negative donors and recipients*

Cross	Donor[a]	Genotype		Recipient	Genotype		Relative frequency[b] of Amp^r recombinants (%)
		ampA	*ampC*		*ampA*	*ampC*	
10	UM100	1	8	H1t14[c]	1	12	0.086
11	UM101	1	9	H1t14	1	12	0.069
12	UM102	1	11	H1t14	1	12	0.111
13	UM103	1	14	H1t14	1	12	0.006
14	UM100	1	8	H1t8	1	3	0.16
15	UM101	1	9	H1t8	1	3	0.13
16	UM102	1	11	H1t8	1	3	0.15
17	UM103	1	14	H1t8	1	3	0.07
18	UM100	1	8	TP1	1	1	0.003
19	UM101	1	9	TP1	1	1	0.018
20	UM102	1	11	TP1	1	1	0.110
21	UM103	1	14	TP1	1	1	0.045
22	G11	+	+	H1t14	1	12	1.0
23	G11	+	+	H1t8	1	3	1.1
24	G11	+	+	TP1	1	1	0.2

[a] Injection order for strains UM100, UM101, UM102, and UM103: origin-*metB-ampA1-purA-fdp-pyrB-thr-leu*. Injection order for strain G11: origin-*purE-leu-thr-ampA1*.

[b] The frequency of Amp^r recombinants is given as a percentage of the recombinants for Thr$^+$ plus Leu$^+$ (crosses 10 to 17, 22, and 23) or Pro$^+$ (crosses 18 to 21 and 24). 100 to 1,000 recombinants of each type were counted.

[c] Strain H1t14 contains a heat-labile β-lactamase (5). The mutant was therefore grown at 44°C, and Amp^r recombinants from crosses with H1t14 as the recipient were selected at 44°C.

was dominant over the wild-type *ampA*$^+$ allele in both an episomal and a chromosomal location. A heterozygote with the combination *ampA*$^+$ *ampC*$^+$/F' *ampA1 ampC12* (SN01 [F'1182]) produced mainly a heat-labile β-lactamase. During growth at 42°C, this strain exhibited a β-lactamase activity comparable to that of a haploid wild-type (*ampA*$^+$ *ampC*$^+$) strain. Moreover, incubation, at 44°C for 1 h, of cell extracts prepared from this heterozygote grown at 28°C destroyed 95% of the β-lactamase activity (Table 5). The remaining activity was similar to that found in the haploid strain SN01 (*ampA*$^+$ *ampC*$^+$). Thus, *ampA1* acted in *cis* but not in *trans*. Strains homozygous for *ampA*$^+$ *ampC*$^+$ or *ampA1 ampC*$^+$ exhibited a β-lactamase activity twice that of the respective haploid strain (Table 4). The lack of interference of *ampA1* on the structural *ampC* gene in the *trans* position was also evident in strain SN02 (F'1182) diploid for *ampA1* and heterozygous for *ampC* (*ampA1 ampC*$^+$/F'*ampA1 ampC12*). At 28°C each enzyme contributed an activity comparable to that mediated by the respective haploid strain (*ampA1 ampC*$^+$ or *ampA1 ampC12*). (Tables 4 and 5).

Owing to its *ampC8* allele, strain SN03 (*ampA1 ampC8*) has virtually no β-lactamase activity. Merodiploid strains carrying *ampC8* on the chromosome were constructed. Surprisingly, all such merodiploids studied exhibited a higher β-lactamase activity than expected (Table 6). The combination *ampA1 ampC8*/F'*ampA1 ampC*$^+$ in strain SN03 (F'1181) resulted in a β-lactamase activity almost twice that of a haploid *ampA1 ampC*$^+$ strain. The ampicillin resistance of this merodiploid strain was increased in parallel with the increase in β-lactamase activity. When the episome was eliminated with aridine orange, haploid clones emerged showing a β-lactamase activity comparable to that of strain SN03. To determine whether the unexpectedly high β-lactamase activity in SN03 (F'1181) was due to an increased activity of episomal enzyme, F'1182 (*ampA1 ampC12*) was introduced into strain SN03

TABLE 4. *β-Lactamase activity and ampicillin resistance of strains haploid or diploid for ampA and ampC*[a]

Strain	Incubation temp (°C)	Genotype on:				Sp act of β-lactamase[b] (U/mg of protein)	Ampicillin resistance[c] (μg/ml)
		Chromosome		Episome			
		ampA	*ampC*	*ampA*	*ampC*		
SN01	37	+	+			0.5	1
SN01 (F'118)	37	+	+	+	+	1.0	2
SN01 (F'1182)	28	+	+	1	12	5.4	NT
SN01 (F'1182)	42	+	+	1	12	0.5	NT
SN02	37	1	+			10.0	11
SN02 (F'118)	37	1	+	+	+	11.7	12
SN01 (F'1181)	37	+	+	1	+	12.0	12
SN02 (F'1181)	37	1	+	1	+	21.0	18
SN02 (F'1182)	28	1	+	1	12	14.8	NT
SN02 (F'1182)	42	1	+	1	12	11.5	NT

[a] Strains and episomes were constructed as described in the text. Haploid and merodiploid strains were grown exponentially at 28, 37, or 42°C in Casamino Acids medium. Uracil (25 μg/ml) was added to the growth medium of haploid strains.

[b] β-Lactamase activity was determined as described in the text.

[c] Ampicillin resistance was determined for single cells as described in the text. NT, Not tested.

TABLE 5. *Heat-labile β-lactamase in merodiploid strains carrying F'1182*[a]

Strain	Genotype on:				Sp act of β-lactamase (U/mg of protein)	
	Chromosome		Episome			
	ampA	*ampC*	*ampA*	*ampC*	28°C	44°C
H1t14	1	12			3.6	<0.06
SN01 (F'1182)	+	+	1	12	5.4	0.5
SN03 (F'1182)	1	8	1	12	7.6	0.06
SN02 (F'1182)	1	+	1	12	14.8	10.2

[a] The merodiploid strains carrying F'1182 were grown in Casamino Acids medium at 28°C. Enzyme extracts were prepared as described in the text. The extracts were divided into two portions, incubated for 1 h at 28 or 44°C, and assayed for β-lactamase activity at room temperature, as described in the text.

TABLE 6. *β-Lactamase activity and ampicillin resistance of merodiploid strains carrying the ampC8 allele*[a]

Strain	Incubation temp (°C)	Genotype on:				Sp act of β-lactamase (U/mg of protein)	Ampicillin resistance (μg/ml)
		Chromosome		Episome			
		ampA	*ampC*	*ampA*	*ampC*		
SN01	37	+	+			0.5	1
SN03	37	1	8			0.03	1
SN03 (F'118)	37	1	8	+	+	1.5	2
SN02	37	1	+			10.0	11
SN03 (F'1181)	37	1	8	1	+	18.6	17
H1t14	28	1	12			3.6	NT
H1t14	42	1	12			0.06	NT
SN03 (F'1182)	28	1	8	1	12	7.6	NT
SN03 (F'1182)	42	1	8	1	12	0.06	NT

[a] Experimental conditions were as described in the text. NT, Not tested.

(*ampA1 ampC8*). This merodiploid showed only the heat-labile activity coded for by the *ampC12* allele. Moreover, the β-lactamase activity found in these cells growing at 28°C was considerably higher than that of the haploid strain H1t14 (*ampA1 ampC12*) (Table 6).

DISCUSSION

The proven β-lactamase structural gene mutations *ampC3* and *ampC12* are, like the *ampA1* mutation, cotransducible with *purA* (5). The five additional β-lactamase-negative mutations studied here showed close linkage to *ampC3* and *ampC12*. The extremely low number of Amp^r recombinants obtained in crosses 10 to 21 (Table 3) suggested that they resulted from intragenic crossovers. All mutants studied gave rise to Amp^r recombinants in crosses with wild-type strains, demonstrating that they still contained the parental *ampA1* allele. It thus appears that all β-lactamase-negative mutants investigated carry lesions in the structural gene for chromosomal β-lactamase, *ampC*. This conclusion was supported by the finding that the *ampC8* allele showed no complementation with *ampC12*.

The tentative order of mutational sites in mutants UM100, UM101, UM102, and UM103 was *ampC8-ampC9-ampC14-ampC11* as judged from crosses 2 to 9 (Table 2). It was also possible to locate the mutations in TP1 (*ampC1*), H1t8 (*ampC3*), and H1t14 (*ampC12*) by using mutants UM100, UM101, UM102, and UM103 as references (Table 3, crosses 10 to 21). The order of mutations studied appeared to be (*ampC1 ampC8*)-*ampC9*-(*ampC12, ampC14*)-*ampC11-ampA1*. Although located in the same cluster, *ampC3* was not strongly linked to any other *ampC* mutation (Fig 1).

The order between the *ampC*, *ampA*, and *purA* loci was deduced from crosses 2 to 5, Table 2, where the majority of Amp^r recombinants obtained were also Pur$^+$. This implied that a single crossover generated an Amp^r Pur$^+$ phenotype, indicating the gene order *ampC-ampA-purA*. The distance between *ampA1* and the *ampC* mutations must be very short compared with that between *ampA1* and *purA*, which is on the order of 0.6 min (1). It can therefore be suggested that no other genes are located between *ampA1* and the structural gene for β-lactamase, *ampC*. It should be emphasized that frequencies of crossover extremely close to a mutation can be influenced by the particular mutation under study (marker-specific effect) (14). Therefore, such frequencies do not always reflect map distance. However, the fact that the order of *ampC* mutations in relation to *ampA1* (crosses 2 to 9, Table 2) was also reflected in the assumed intragenic crosses (10 to 13 and 18 to 21, Table 3) adds some strength to our data.

Dominance studies were performed to obtain further information about the nature of *ampA1*. In diploid situations, *ampA1 ampC*$^+$ was dominant over *ampA*$^+$ *ampC*$^+$. However, when F'1182 (*ampA1 ampC12*) mediating a heat-labile β-lactamase was introduced into the wild-type strain, SN01 (*ampA*$^+$ *ampC*$^+$), the wild-type level of β-lactamase activity was found at the restrictive temperature. Moreover, when the same episome was introduced into an *ampA1 ampC*$^+$ strain, heat-labile and heat-stable β-lactamase were formed in expected proportions. Thus, *ampA1* was active in *cis* but not in *trans*. Available data may be interpreted such that *ampA1* increases the efficiency of transcription of the neighboring *ampC*. Since these loci are very closely linked, *ampA* may be the promoter or operator region for *ampC*. This is supported also by the earlier finding that *ampA1*-carrying strains apparently produce the wild-type β-lactamase protein (10). An evi-

dent gene dosage effect was found in all strains diploid for *ampA* and *ampC*. A gene dosage effect is also known for the periplasmic β-lactamase mediated by an R-plasmid (15). It is interesting that no such gene dosage effect was found for an outer membrane protein (7). This was suggested to be due to a limitation of space in the outer membrane for this protein. Unincorporated protein was supposed to act as an autorepressor of its own biosynthesis. The chromosomal β-lactamase is a periplasmic enzyme (10). Any space limitation for this enzyme is therefore unlikely when it is produced from more than one gene copy. Merodiploid strains carrying the *ampC8* allele on the chromosome exhibited a surprisingly high β-lactamase activity, apparently resulting from derepression of the episomal *ampC* allele. One possible interpretation of this result is that the cytoplasmic portion of the β-lactamase acts as an autorepressor in a way similar to that proposed for protein II* of the outer membrane (7). If the *ampC8* gene product is inactive not only catalytically, but also in this negative feed back system suggested, the episomal β-lactamase in the cytoplasm would be titrated out by two operons, leading to an increased level of β-lactamase synthesis. However, at present we cannot exclude the possibility that *ampC8* either increases the number of episome copies per cell or alters the rate of β-lactamase secretion.

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