ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 1985, p. 21–27
0066-4804/85/070021-07$02.00/0

Vol. 28, No. 1

Genetic Analysis of Spontaneous Resistance to Ampicillin in *Neisseria gonorrhoeae*

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Received 19 September 1984/Accepted 16 April 1985

Step-wise intrinsic resistance to ampicillin in *Neisseria gonorrhoeae* was analyzed genetically by DNA-mediated transformation experiments. A first-step ampicillin-resistant (Amp^r_1) mutant and a second-step ampicillin-resistant (Amp^r_2) mutant generated during sequential selection were used in these studies. Each selection step was accompanied by an approximate twofold increase in resistance. Four *amp* alleles were found to account for full resistance of the Amp^r_2 phenotype. All four *amp* alleles lie among a cluster of genes which code for ribosomal functions. This region has the map order *rif str fus tet cam*. First-step resistance was caused by two *amp* alleles, *ampA2* and *ampB1*, neither of which independently caused detectable ampicillin resistance. Outcrossing of the *ampA2* or the *ampB1* mutation resulted in wild-type susceptibility to ampicillin. Mapping studies indicate that *ampB1* lies between *str* and *fus*, whereas *ampA2* lies to the right of *cam*. Second-step resistance required two mutations, *ampC3* and *ampD4*, in addition to *ampB1* and *ampA2*. Transformation of *ampC3* to $ampC3^+$ in an Amp^r_2 mutant resulted in the Amp^r_1 phenotype. Both *ampC3* and *ampD4* showed transformation linkage to *rif* and *str*. *ampC3* was positioned at a site between *rif* and *str*. *ampD4* apparently occupied a site, outside of the *rif-str* region, proximal to *rif* and distal to *str*. We postulate the gene order to be *ampD rif ampC str ampB fus tet cam ampA*.

Intrinsic resistance to penicillin, ampicillin, and other β-lactam antibiotics in bacteria is acquired by mutation in one or more chromosomal genes (1). Extrinsic resistance to these antibiotics can be under the control of β-lactamase genes of plasmid or chromosomal origin (11). Genetic studies of intrinsic ampicillin resistance in *Streptococcus pneumoniae* showed that resistance to increasing levels of ampicillin resulted from the additive effects of mutations at several independent chromosomal loci (3). From studies of intrinsic penicillin resistance in *Neisseria gonorrhoeae*, three genes, *penA* (7, 12, 16), *penB* (16), and *pem* (18), have been reported to confer cross-resistance to ampicillin. Mutations in *penA* result in low-level resistance to penicillin, ampicillin, and certain other penicillins but not to other drugs (7, 12, 16). Mutations at *penB* or at *pem* confer no resistance independently (15, 16, 18). *penB* mutations increase the nonspecific effects expressed by *mtr*, which include low-level resistance to penicillin and tetracycline (15) and slightly increased resistance to ampicillin and chloramphenicol (16). Mutations in *pem* and *penA* in combination additively increase resistance to penicillin and ampicillin (16, 18). Mapping studies indicate that *penB* is linked to a cluster of genes which code for resistance to streptomycin, fusidic acid, tetracycline, chloramphenicol, erythromycin, and spectinomycin (12, 15, 16).

The genes *penA* and *pem* show no cotransformation with any of the genes within this cluster but do exhibit 90% cotransfer with each other (18).

This report presents evidence for multiple chromosomal genetic loci which cause ampicillin resistance in *N. gonorrhoeae* and are distinct from loci previously described.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in these studies are listed in Table 1. All strains were stored at −70°C in

TABLE 1. Strains used

Strain	Phenotype[a]	Origin
2686	Parental	D. S. Kellogg
JN25	Str^r	Spontaneous Str^r mutant of 2686
JN26	Amp^r_1	Spontaneous Amp^r mutant of 2686
JN27	Amp^r_2	Spontaneous Amp^r mutant of JN26
JN28	Amp^r_3	Spontaneous Amp^r mutant of JN27
JN29	Amp^r_1 Str^r	Recombinant from JN25 × JN26
JN30	Amp^r_2 Str^r	Spontaneous Str^r mutant of JN27
JN31	Tet^r	Recombinant from JN38 × 2686
JN32	Amp^r_1 Tet^r	Recombinant from JN38 × JN26
JN33	Fus^r	Spontaneous Fus^r mutant of 2686
JN34	Amp^r_1 Fus^r	Spontaneous Fus^r mutant of JN26
JN35	Rif^r	Recombinant from JN36 × 2686
JN36	Amp^r_2 Rif^r	Spontaneous Rif^r mutant of JN27
JN37	Amp^r_2 Fus^r	Spontaneous Fus^r mutant of JN27
JN38	Amp^r_2 Tet^r	Spontaneous Tet^r mutant of JN27
JN45	Amp^r_2 Rif^r Str^r	Spontaneous Rif^r mutant of JN30
JN49	Amp^r_1 Amp^r_2 Rif^r Str^r	Recombinant JN45 × JN26
JN55	Amp^r_1 Amp^s_2 Rif^r	Recombinant JN45 × JN26
JN56	Amp^r_1 Amp^s_2 Rif^r	Recombinant JN45 × JN26
JN60	Amp^r_1 Amp^r_2 Rif^r Tet^r	Recombinant JN32 × JN49
JN67	Cam^r	Spontaneous Cam^r mutant of JN27
JN68	Cam^r	Recombinant JN67 × 2686
JN81	Amp^r_1 Cam^r	Spontaneous Cam^r mutant of JN26
JN82	Amp^r_1 Fus^r Str^r	Recombinant JN25 × JN34
JN84	Amp^s_1 Fus^r Str^r	Recombinant JN25 × JN34
JN85	Amp^s_1 Fus^r Str^r	Recombinant JN25 × JN34
JN86	Amp^s_1 Tet^r Fus^r Str^r	Recombinant JN31 × JN82
JN87	Amp^s_1 Tet^r Fus^r	Recombinant JN33 × JN32

[a] Str, Streptomycin; Amp, ampicillin; Tet, tetracycline; Fus, fusidic acid; Rif, rifampin; Cam, chloramphenicol.

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TABLE 2. Antibiotic susceptibility of *N. gonorrhoeae* mutants

Strain (phenotype)	MIC (μg/ml)[a]						
	Amp	Pen	Tet	Ery	Cam	Fus	Rif
2686 (Parent)	0.025	0.006	0.085	0.120	0.300	0.110	0.025
JN26 (Amp^r_1)	0.050	0.010	0.120	0.130	0.300	0.110	0.025
JN27 (Amp^r_2)	0.095	0.016	0.110	0.120	0.300	0.110	0.020
JN28 (Amp^r_3)	0.200	0.014	0.110	0.120	0.350	0.110	0.025

[a] MIC is the lowest concentration of antibiotic that produces at least a 90% inhibition of colony-forming activity. Amp, Ampicillin; Pen, penicillin; Tet, tetracycline; Ery, erythromycin; Cam, chloramphenical; Fus, fusidic acid; Rif, rifampin.

GCBB-DS (gonococcal medium base broth with defined supplements) containing 20% (vol/vol) glycerol (14).

Media, culture conditions, and enzyme assays. Cultures were routinely grown on GCBA-DS (gonococcal medium base agar [Difco] plus defined supplements) or in GCBB-DS at 36°C in a humidified atmosphere containing 5% CO_2 (17). By using the criteria established by Kellogg et al. (5), colony types were determined by use of an AO Cycloptic dissecting microscope (American Optical Corp.) or an Olympus model SC stereo microscope (Olympus Corp. of America). All antibiotics were added to molten agar medium held at 50°C before pouring of the plates. With the exception of media containing streptomycin and ampicillin, antibiotic-containing media were used within 1 to 4 days of preparation. Medium containing ampicillin was used within 24 h. Medium containing streptomycin was stored for as long as 2 months at 4°C. Plates were routinely allowed to dry overnight at room temperature and subsequently stored at 4°C. β-lactamase was assayed by the method of Catlin (4).

Selection of antibiotic-resistant mutants. A procedure involving the sequential subculture of gonococci on media containing increasing concentrations of antibiotic was used for selecting multi-step ampicillin-resistant mutants. First-step (Amp^r_1) mutants were obtained as follows. A heavy cell suspension of parental strain 2686, 10^9 CFU/ml, was prepared, and 0.1-ml samples were spread onto 15 to 20 GCBA-DS agar plates containing 0.04 μg of ampicillin per ml. Emergent colonies were first tested for resistance by streaking onto the same medium. MICs were then determined for isolates that exhibited typical T_1 or T_2 colonial morphology. An isolate showing first-step resistance to 0.05 μg of ampicillin per ml was used for the selection of mutants with increased resistance to ampicillin above the first-step level, i.e., second-step resistance. After the procedure described above, selection at 0.08 μg of ampicillin per ml yielded second-step (Amp^r_2) mutants resistant to an ampicillin concentration of 0.095 μg/ml. Third-step ampicillin-resistant (Amp^r_3) mutants were selected in media containing 0.15 μg of ampicillin per ml. These mutants subsequently exhibited resistance to an ampicillin concentration of approximately 0.20 μg/ml.

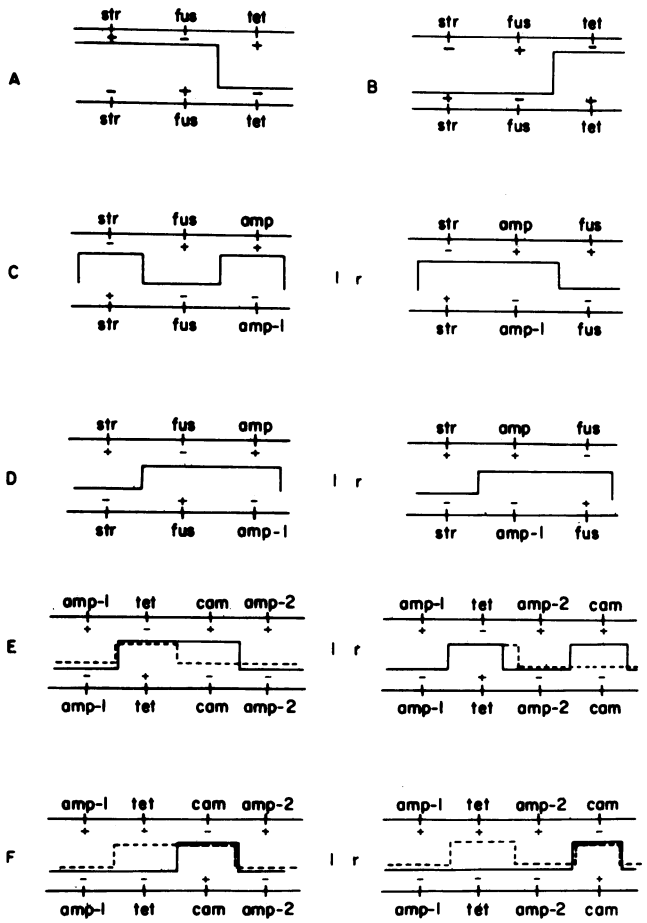

FIG. 1. Schematic crosses to map genes for first-step ampicillin resistance. (A) Selection to bias recombination toward the left of *tet*. (B) Selection to bias recombination toward the right of *fus*. (C) The first cross of a three-factor reciprocal cross designed to order *amp-1* relative to *fus* and *str*. Left (l) and right (r) figures represent the two possible gene orders being tested. (D) The reciprocal cross of (C). (E and F) Four factor crosses to order *amp-2* relative to *tet* and *cam*. Left (l) and right (r) figures represent the two possible gene orders.

TABLE 3. Cotransformation analysis of first-step ampicillin resistance

Cross	Donor (phenotype)	Recipient (phenotype)	Selected phenotype	Unselected phenotype	No. of transformants screened	No. of transformants with unselected phenotype	Frequency of cotransformation
A	JN29 (Str^r Amp^r_1)	2686 (Str^s Amp^s)	Str^r	Amp^r_1	465	ND[a]	
			Amp^r_1		ND	ND	
B	JN25 (Str^r Amp^s)	JN26 (Str^s Amp^r_1)	Str^r	Amp^s	543	12	0.022
C	JN32 (Tet^r Amp^r_1)	2686 (Tet^s Amp^s)	Tet^r	Amp^r_1	546	ND	
			Amp^r_1		ND	ND	
D	JN31 (Tet^r Amp^s)	JN26 (Tet^s Amp^r_1)	Tet^r	Amp^s	404	47	0.116
E	JN31 (Tet^r Amp^s)	JN34 (Fus^r Amp^r_1)	Tet^r	Fus^s	484	273	0.564
			Tet^r	Amp^s	484	61	0.126

[a] ND, None detectable.

TABLE 4. Genetic mapping of the *amp-2* allele

Cross	Donor (genotype)	Recipient (genotype)	Selected phenotype	Unselected phenotype(s)	Marker ratio	Frequency	Frequency of cotransformation
A	JN31 (*tet-1*)	JN81 (*amp-1 amp-2 cam-2*)	Tet^r	Amp^s	Amp^s/Tet^r	579/810	0.715
				Cam^s	Cam^s/Tet^r	579/810	0.715
				Cam^r Amp^s	Amp^s/Cam^r Tet^r	177/231	0.766
					Amp^r_1/Cam^r Tet^r	54/231	0.234
				Amp^r Cam^s	Cam^s/Amp^r Tet^r	177/231	0.766
					Cam^r/Amp^r Tet^r	54/231	0.234
B	JN68 (*cam-1*)	JN32 (*amp-1 amp-2 tet-1*)	Cam^r	Amp^s	Amp^s/Cam^r	771/912	0.845
				Tet^s	Tet^s/Cam^r	782/912	0.857
				Amp^r Tet^s	Tet^s/Amp^r Cam^r	37/41	0.902

Spontaneous mutants resistant to the reference antibiotics used in this study were selected by spreading 0.1 ml of a heavy cell suspension (10^9 CFU/ml) onto GCBA-DS agar plates supplemented with the appropriate antibiotics. The concentrations of antibiotics used in selection for each reference mutation were tetracycline (0.25 μg/ml), rifampin (0.3 μg/ml), fusidic acid (0.1 μg/ml), and streptomycin (500 μg/ml).

Determinations of MIC. The MIC was defined as the lowest concentration of antibiotic to produce 90% inhibition of colony-forming activity after 72 h of incubation. Cells were grown on GCBA-DS agar plates for 18 to 20 h, harvested in GCBB-DS broth, and suspended to turbidity equivalent to a concentration of 2×10^3 to 4×10^3 CFU/ml. Samples (0.1 ml) of the cell suspension were then spread onto plates containing various concentrations of antibiotics and onto control plates containing no antibiotic. After incubation at 36°C for 72 h, the MIC was recorded.

DNA isolation and transformation. Transforming DNA was extracted from donor strains by a modification of a procedure described by Sparling (14). Cell lysis usually occurred within 10 to 20 min after the addition of powdered sodium lauryl sulfate. The DNA fibers collected after three alcohol precipitations were dissolved in sodium citrate buffer and used for all transformation experiments. DNA concentrations were estimated by the diphenylamine method of Burton (2). Transformation experiments were carried out by the procedure described by Sparling (14). Cotransformation of the unselected resistant phenotypes was determined by replica-plating the transformed colonies to antibiotic-free agar plates and then to media containing the unselected antibiotic. Transformed colonies were replicated by transferring each individual colony to the appropriate medium with sterile toothpicks. Cotransformation frequencies were expressed as the ratio of transformants with the unselected phenotype to the total number of colonies screened for the selected phenotype.

Antibiotics. Ampicillin (polycillin-N) was obtained from Bristol Laboratories, Syracuse, N.Y. Penicillin-G (sodium salt), erythromycin, and tetracycline were kindly provided by George B. Whitfield, The UpJohn Co., Kalamazoo, Mich. Rifampin and chloramphenicol were obtained from Sigma Chemical Co., St. Louis, Mo. Fusidic acid sodium salt (Fusidin) was a gift from W. O. Godtfredsen, Leo Pharmaceutical Products, Ballerup, Denmark. Streptomycin sulfate was obtained from Pfizer Laboratories Div., New York, N.Y.

Other chemicals used were reagent grade or of the highest purity commercially available.

RESULTS

Characterization of ampicillin-resistant phenotypes. The selection of ampicillin-resistant (Amp^r) mutants in three selection steps was characterized by a twofold increase in resistance at each step. The apparent mutation frequency was in the range of 10^{-9} to 10^{-10}, and the step-wise pattern was consistent with observations made in other genera (3, 11, 13). Amp^r_1, Amp^r_2, and Amp^r_3 mutants were tested for ampicillin resistance and cross-resistance to other antibiotics (Table 2). Other than a slight increase in resistance to penicillin and to tetracycline in the first step, susceptibility to other antibiotics tested remained unchanged. This suggested that the mutations causing multiple-step ampicillin resistance, and in particular the low-level tetracycline resistance, do not involve the *mtr* locus (9, 10), which confers increased resistance to all of the antibiotics tested except ampicillin. β-lactamase activity, assayed by the method of Catlin (4), was not detectable.

Cotransformation analysis of first-step ampicillin resistance. Amp^r_1 mutants were characterized genetically by transformation with isogenic strains. No Amp^r transformants were recovered by direct or indirect selection when DNA from Amp^r_1 Str^r or Amp^r_1 Tet^r mutants was used to transform the Amp^s parental strain 2686 (Table 3, crosses A and C). However, Amp^r_1 mutants were transformed to Amp^s by cotransformation with Str^r at a frequency of 2.2% or Tet^r at frequencies of 11.6 and 12.6% (Table 3, crosses B, D, and E). Fus^s cotransformed with Tet^r at a frequency of 56.4%. Since Amp^s but not the Amp^r_1 phenotype could be transferred in a single transformation event, we inferred that spontaneous mutation to first-step ampicillin resistance may involve two or more genetic sites with low or no transformation linkage to each other.

On the tentative assumption that two "resistance" alleles caused first-step ampicillin resistance, experiments were designed to determine their locations relative to the known positions of the antibiotic markers *str*, *fus*, and *tet*, which have been previously mapped by Sparling et al. (15). The crosses shown in Fig. 1A and B were designed to measure the cotransfer of markers for Amp^s with regions of the chromosome to the left of *tet* to include *fus* but exclude *tet* and to the right of *tet* to include *tet* but exclude *fus*. In cross A, with Tet^r in the recipient, double selection for Fus^r Tet^r biased selection for only those Amp^s transformants which incorporated the donor chromosome region to the left of *tet* (Fig. 1A). In cross B, with Fus^r in the recipient, selection for Fus^r Tet^r biased selection of transformants which incorporated the donor chromosome to the right of *fus* (Fig.

TABLE 5. Cotransformation frequency of mutations causing second-step ampicillin resistance

Cross	Donor (phenotype)	Recipient (phenotype)	Selected phenotype	Unselected phenotype	No. of transformants screened	No. of transformants with unselected phenotype	Frequency of cotransformation
1	JN30 (Amp^r_2 Str^r)	JN33 (Fus^r)	Str^r	Fus^s	765	439	0.574
				Amp^r_2	765	0	0
2	JN30 (Amp^r_2 Str^r)	JN34 (Amp^r_1 Fus^r)	Str^r	Fus^s	414	242	0.585
				Amp^r_2	414	8	0.019
3	JN37 (Amp^r_2 Fus^r)	JN25 (Str^r)	Fus^r	Str^s	531	369	0.695
				Amp^r_2	531	0	0
4	JN37 (Amp^r_2 Fus^r)	JN29 (Amp^r_1 Str^r)	Fus^r	Str^s	411	278	0.676
				Amp^r_2	411	0	0
5	JN38 (Amp^r_2 Tet^r)	JN25 (Str^r)	Tet^r	Str^s	956	423	0.443
				Amp^r_2	956	0	0
6	JN38 (Amp^r_2 Tet^r)	JN29 (Amp^r_1 Str^r)	Tet^r	Str^s	840	375	0.446
				Amp^r_2	840	0	0
7	JN35 (Rif^r)	JN25 (Str^r)	Rif^r	Str^s	414	92	0.222
8	JN25 (Str^r)	JN35 (Rif^r)	Str^r	Rif^s	405	43	0.106

1B). The results from cross A indicate that a site to the left can cause Amp^s with a cotransformation frequency of 99.7% with *fus*. A second site causing Amp^s was revealed by cross B to lie to the right of *tet* with a cotransformation frequency of 30.6% with *tet*. From these results an apparent gene order is *str amp-1 fus tet amp-2*.

Mapping of first-step ampicillin resistance alleles. A three-factor reciprocal cross was designed to position the *amp-1* allele relative to *fus* and *str*. The position of *amp-1* relative to *fus* and *str* was deduced from the frequency of cotransformation of Amp^s with *str* or with *fus* when recombination occurred between *fus* and *str*. Fig. 1C depicts the expected recombinational events for the two possible gene orders indicated. Similarly, recombinational events for the reciprocal cross are represented by Fig. 1D. If the gene order were *str fus amp-1*, then the appearance of Amp^s should be infrequent in cross C, owing to a double recombinational event (probably less than 1%), and frequent in cross D as a consequence of a single recombinational event. However, if the gene order were *str amp-1 fus* then the appearance of Amp^s should be frequent in both crosses. The appearance of Amp^s among Str^r Fus^r transformants occurred at a frequency of 46% in cross C and 84% in cross D. These data support a gene order of *str amp-1 fus*.

A second locus involved in first-step ampicillin resistance, *amp-2*, was positioned relative to *tet* and *cam*. Standard three-factor reciprocal cross analyses could not be done with the markers available because no transformants were viable when Tet^r and Cam^r were simultaneously selected phenotypes. Therefore, four-factor crosses of single selected and multiple unselected markers were used to order *amp-2* relative to *cam* (Fig. 1E and F and Table 4). In cross E (Fig. 1) with Tet^r (tet^-) as a single selected marker, Amp^s (amp^+) and Cam^s (cam^+) cotransformed at a frequency of 71.5%. The frequency for Cam^s reflects probable distance between the *tet* and *cam* alleles; however, the frequency for Amp^s and Tet^r is higher than a frequency which reflects the probable separation between *tet* and *amp-1* or *amp-2*. This may be explained if a gene for Amp^s lies to either side of *tet*, in which case the frequency with which Amp^s cotransforms with Tet^r is the sum of the frequencies for *amp-1* and *amp-2* each to cotransform with *tet*. Among the Tet^r transformants which were Cam^r in cross A, Amp^s appeared with a frequency of 76.6%, whereas Amp^r appeared at a 23.4% frequency (Table 4). These frequencies indicate that single recombinational events can account for the phenotypes observed. Among the Tet^r transformants which were Amp^r, the Cam^s and Cam^r phenotypes cotransformed at frequencies consistent with single recombinational events (Table 4). Fig. 1E is a diagram of the recombinational events expected given the two possible gene orders indicated. If the gene order were *amp-1 tet amp-2 cam*, then the phenotype ratio Cam^s/Amp^r Tet^r would be very low to reflect a double recombinational event. The frequency of 0.766 was high and therefore consistent with the gene order *amp-1 tet cam amp-2*.

The reciprocal cross, Fig. 1F, yielded a high frequency of Amp^s in cotransformation with the selected Cam^r phenotype (Table 4, cross B). Appearance of Tet^s phenotypes among the selected Cam^r recombinants occurred at a frequency of 85.7%. This frequency reflects the proximity of *tet* to *cam*. The frequency of appearance of Tet^s among Cam^r Amp^r transformants was 90.2%. This frequency connotes a single recombinational event. In the diagrams of recombinational possibilities related to gene order, single recombinational events suggest a gene order of *amp-1 tet cam amp-2* (Fig. 1F). Therefore, the results of both crosses E and F (Fig. 1) are consistent with the suggested gene order. We suggest that the ampicillin-susceptible allele of *amp-1* be labeled *ampB* and the susceptible allele of *amp-2* be labeled *ampA*.

Reconstruction of the Amp^r_1 phenotype. The combined effect of the *amp-1* and *amp-2* alleles to produce the Amp^r_1 phenotype was directly demonstrated by transformation. Strains JN86 and JN87 with the genotypes str^- $ampB1^-$ fus^- tet^- $ampA^+$ and str^+ $ampB^+$ fus^- tet^- $ampA2^-$, respectively, were constructed from appropriate crosses. From a cross between these two Amp^s strains, JN86 (donor) × JN87, with Str^r as the selected phenotype, first-step ampicil-

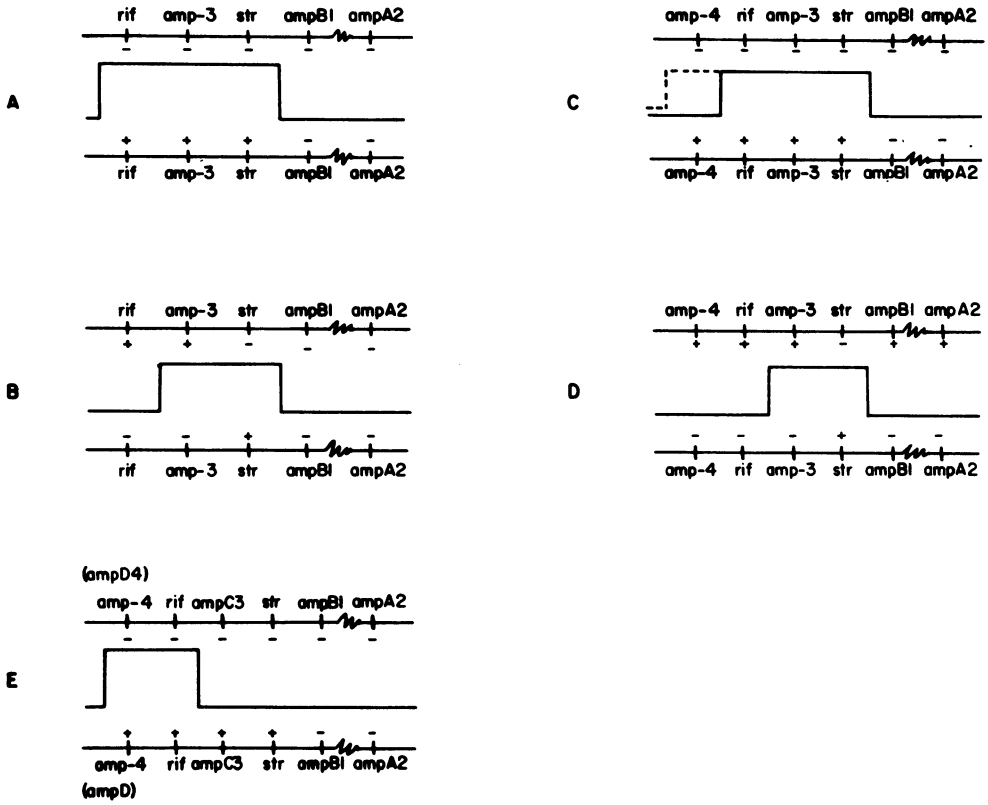

FIG. 2. Designs of crosses for genetic mapping of *amp-3* and *amp-4*. (A) Hypothetical assignment of *amp-3*. Double selection for donor *rif-str* yields approximately 30% Rif^r Str^r Amp^r_2 expression. (B) Double selection for donor *str*-recipient *rif* yields transformants (outcrossed by a single amp^+ allele) of the phenotype Amp^r_1. (C) Double selection for donor *rif-str* yields transformants with genes to the left of *str* to include *amp-3 rif* and *amp4*. (D) Recombinational events showing cotransfer of *str amp-3*$^+$ or *str ampB*$^+$. (E) Construction of the genotype *ampA2 ampB1 ampC*$^+$ *amp-4* (Amp^s_2).

lin resistance cotransformed at a frequency of 90.7%. The level of ampicillin resistance in the reconstructed strain permitted growth on 0.04 μg of ampicillin per ml, the same as the Amp^r_1 mutants. This high frequency of cotransformation strongly supports an interpretation that JN87 harbors one of two alleles necessary for first-step ampicillin resistance and that a single allele near *str* is also required for this resistance.

Mapping of second-step ampicillin resistance. Second-step ampicillin resistance apparently cotransformed with *str* and only to strains that expressed the first-step resistance to ampicillin (Table 5, crosses 1 and 2). Whereas the *str* marker was linked by cotransformation to *rif*, *tet*, and *fus* (Table 5, crosses 3 through 6), the allele(s) for the Amp^r_2 phenotype cotransformed only with *str* and with a very low frequency. The *rif* and *str* loci are sufficiently close (Table 5, crosses 7 and 8) that a single marker with low linkage to *str* and unlinked to *fus* or *tet* would be expected to cotransform with *rif* at a higher frequency than with *str*. However, we observed a comparably low cotransformation frequency (0.007) of Amp^r_2 with *rif*. We considered that second-step ampicillin resistance expression might derive from two genes, one to either side of *rif*. A requirement for both genes to cotransform with *str* or *rif* could account for the very low frequencies observed. We designed crosses to test this hypothesis (Fig. 2).

If a single gene between *rif* and *str* were responsible for second-step ampicillin resistance, then the double selection of *rif* and *str* transformed from an Amp^r_2 donor to an Amp^r_1

TABLE 6. Evidence for two alleles required for second-step ampicillin resistance

Cross	Donor (genotype)	Recipient (genotype)	Selected marker	Unselected marker (phenotype)	No. of transformants screened	No. of unselected markers	Frequency of cotransformation
A	JN45 (*ampB1 ampA2 amp-3 amp-4 rif-2 str-2*)	JN26 (*ampB1 ampA2*)	*rif-2 str-2*	*amp-3 amp-4* (Amp^r_2)	540	155	0.287
B	JN25 (*str-1*)	JN60 (*ampB1 ampA2 amp-3 amp-4 rif-2 tet-1*)	*str-1 rif-2*	*amp-3*$^+$ (Amp^r_1)	270	47	0.174
C	JN29 (*ampB1 ampA2 str-1*)	JN60 (*ampB1 ampA2 amp-3 amp-4 rif-2 tet-1*)	*str-1 rif-2*	*amp-3*$^+$ (Amp^r_1)	270	67	0.248

TABLE 7. Independent cotransformation of *ampC3* and mapping of genes responsible for second-step ampicillin resistance

Cross	Donor (genotype)	Recipient (genotype)	Selected marker(s)	Unselected marker	No. of transformants screened	No. of transformants with unselected phenotype	Frequency of cotransformation
A	JN45 (*ampB1 ampA2 ampC3 amp-4 rif-2 str-2*)	JN55 (*ampB1 ampA2 ampC3*$^+$ *amp-4 rif-2*)	*str-2*	*ampC3*$^+$	270	0	0
				ampC3	270	260	0.963
B	JN45 (*ampB1 ampA2 ampC3 amp-4 rif-2 str-2*)	JN56 (*ampB1 ampA2 ampC3*$^+$ *amp-4 rif-2*)	*str-2*	*ampC3*$^+$	270	0	0
				ampC3	270	262	0.970
C	JN45 (*ampB1 ampA2 ampC3 amp-4 rif-2 str-2*)	JN26 (*ampB1 ampA2 ampC3*$^+$ *amp-4*$^+$)	*rif-2*	*ampC3*$^+$ *str*$^+$	270	120	0.440
			str-2	*ampC3 amp-4*	270	37	0.137
			rif-2 str-2	*ampC3 amp-4*	261	22	0.084
				ampC3 amp-4	251	75	0.300

recipient should yield a fraction of Amp^r_2/Rif^r Str^r transformants that approached 100% (Fig. 2A). Likewise, an Amp^r_2 Rif^r recipient transformed with an Amp^s Str^r (data not shown) or Amp^r_1 Str^r donor should outcross a single gene for second-step resistance by selection for Rif^r Str^r (Fig. 2B). Transformation of strain JN26 (Amp^r_1) by DNA from strain JN45 (Amp^r_2) by double selection for *rif* and *str* resulted in the cotransformation of the Amp^r_2 at a frequency of 28.7% (Table 6, cross A, and Fig. 2C). This frequency represents a 15- to 30-fold increase over results obtained by cotransformation with either *rif* or *str* as a single selected marker. This dramatic increase implied that the region between *rif* and *str* could account, at least in part, for second-step resistance. However, since the frequency of appearance of Amp^r_2 did not approach 100% in the doubly selected recombinants, a region left of *rif* was also implicated in expression of second-step resistance (Fig. 2C). The inverse crosses with selection for recipient *rif* and donor *str* yielded comparable frequencies of Amp^r_2 (Table 6, crosses B and C, Fig. 2B or D). These data then confirm the presence of a locus between *rif* and *str* that is involved in expression of second-step resistance. We suggest *ampC* as a name for this locus between *rif* and *str*.

In an effort to determine whether a fourth allele was involved in expression of second-step ampicillin resistance, we designed selections to construct a strain to contain mutations *ampA2 ampB1* and a putative fourth allele (*amp-4*) but not the *ampC3* (*amp-3*). The desired recombination pattern to achieve the strain construction is shown in Fig. 2E, and the results are presented in Table 7. The cross between JN45 (donor) and JN26 yielded Rif^r transformants without second-step ampicillin resistance but with Str^s and first-step ampicillin resistance. The frequency for the Amp^r_1 Str^s/Rif^r phenotype was 44%. If a locus, *amp-4*, required for second-step resistance lay to the left of and near *rif*, then some of these transformants would be expected to lack *ampC3* but to contain the *amp-4* allele. Two transformants with the appropriate phenotypes from this cross were labeled JN55 and JN56.

If strains JN55 and JN56 contained three of four alleles necessary for expression of second-step ampicillin resistance and if the missing allele were *ampC*, then we reasoned that second-step ampicillin resistance should cotransform with *str* at a frequency much higher than expected from the previous crosses which required two alleles to transform. The results of crosses A and B between strains JN45 and JN55, and JN45 and JN56 clearly supported the four allele hypothesis (Table 7). The Amp^r_2 phenotype appeared at a cotransformation frequency of 96% and 97% respectively. Control crosses showed that DNA from JN45 cotransformed second-step ampicillin resistance with either *rif* or *str* at low frequencies, and selection for *rif-str* recombination raised the frequency to 30%. We suggest *ampD* as a name for the locus to the left of *rif*.

These data provide evidence that four alleles are involved in expression of first- and second-step ampicillin resistance. Mapping evidence is consistent with the gene order shown in Fig. 3.

DISCUSSION

These results indicate that ampicillin resistance in *N. gonorrhoeae* arises by a series of mutations in multiple genes distinct from those previously mapped (6, 8, 11, 15, 16). Mutation frequencies from initial selection were 10^{-9} to 10^{-10}, although the first-step resistance was shown to derive from two mutation sites. The apparent high frequency for a double mutational event might be explained by two single sequential events that occurred as a consequence of transfer onto the selective medium for clonal purification after the initial isolation. Alternatively, the region in which these mutations lie might have unusually high mutability. Polygenic inheritance is a common mechanism for the development of resistance to antibiotics and has been demonstrated for penicillin and tetracycline resistance in the gonococcus (6, 15, 16).

The first increment of ampicillin resistance requires mutation in both *ampA* and *ampB* for detection. The slight increase in tetracycline resistance associated with the *ampA2 ampB1* genotype is apparently due to the *ampA2* mutation. The *penB* locus, which may code for ampicillin resistance when *mtr* is present, has been reported to lie in the same region as *ampA*. However, *penB* and *ampA* appear to be different genes based on a comparison of our mapping data with that of Sparling (15). The cotransformation fre-

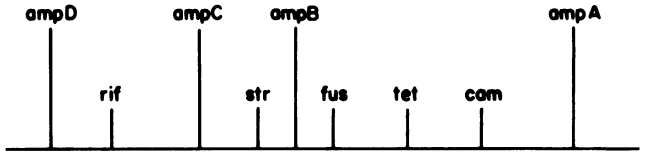

FIG. 3. Probable map positions of four ampicillin-resistance genes. The *amp* genes were mapped relative to the locations of the drug resistance genes, *rif*, *str*, *fus*, *tet*, and *cam* as reported by Sparling et al. (16) and Spratt et al. (17).

quency of *penB* with *cam* was 10% (15), whereas *ampA* and *cam* cotransformed at 80 to 90% frequency. In addition, ampicillin resistance associated with *penB* is expressed only in strains containing the *mtr* locus (15). The *tem* locus, which apparently lies in the vicinity of the *ampB* and *ampC* loci (18), likewise appears not to be functionally allelic to *ampB* or *ampC*. The only reported effects of *tem* are to increase resistance conferred by *pem* and *tet* mutations (18).

Ampicillin resistance above the first-step requires mutations in both *ampC* and *ampD* in addition to the first-step lesions. The concomitant increase in penicillin resistance of about 50% by mutation in *ampC ampD* was the only observed change in resistance to the antibiotics tested other than ampicillin. This increase may be attributable to a cross-resistance to penicillin and may involve the same target site.

The newly identified genes were positioned relative to neighboring reference genes by multifactor reciprocal crosses. In some mapping designs, three-factor reciprocal crosses would not work because the selection requiring two ribosomal alterations, e.g., Tet^r and Cam^r yielded no viable transformants. Positions of the *amp* alleles were deduced primarily from the relative frequencies of cotransformation with neighboring reference genes. In mapping the *amp* alleles, we were always aware of the possible cotransfer of neighboring *amp* alleles which could increase expected cotransformation frequencies. However, the distances separating the *amp* genes, e.g., *ampA* and *ampB* were sufficient such that their cotransfer, presumably, would be rare. This hypothesis is supported indirectly by experiments in which the cotransfer of two ampicillin resistance alleles was usually nondetectable or detected only at low frequencies. In addition, selection procedures designed to bias the selection of transformants incorporating specific regions of the chromosome helped to define the transfer of single *amp* genes. The map positions for the reference genes *str*, *rif*, *fus*, *tet*, and *cam* used in our studies with *N. gonorrhoeae* 2686 were in close agreement with those published for strain FA19 (15).

These studies report an example not found in the literature in which resistance of a multi-step mutant results from the combined effects of a series of mutations, none of which confers resistance independently. These mutations permitted establishment of the gene order *ampD rif ampC str ampB fus tet cam ampA*.

ACKNOWLEDGMENTS

This research was partially supported by Public Health Service Minority Biomedical Research Support grant RR08037 from the National Institutes of Health, Division of Research Resources. J.H.J. is a recipient of Research Career Development award CA00533 from the National Cancer Institute.

LITERATURE CITED

1. **Benveniste, R., and J. Davies.** 1973. Mechanisms of antibiotic resistance in bacteria. Annu. Rev. Biochem. **43**:471–506.
2. **Burton, K.** 1956. A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. **62**:315–323.
3. **Butler, L. O., and M. B. Smiley.** 1970. Characterization by transformation of an ampicillin-resistant mutant of *Pneumococcus*. J. Gen. Microbiol. **61**:189–195.
4. **Catlin, B. W.** 1975. Iodometric detection of *Haemophilus influenzae* beta-lactamase: rapid presumptive test for ampicillin resistance. Antimicrob. Agents Chemother. **7**:265–270.
5. **Kellogg, D. S., Jr., W. L. Peacock, Jr., W. E. Deacon, L. Brown, and C. I. Pirkle.** 1963. *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. J. Bacteriol. **85**:1274–1279.
6. **Maier, T. W., H. R. Beilstein, and L. Zubrzycki.** 1974. Multiple antibiotic resistance in *Neisseria gonorrhoeae*. Antimicrob. Agents Chemother. **6**:22–28.
7. **Maier, T. W., P. Warner, L. Zubrzycki, and M. Chila.** 1977. Identification of drug resistance loci in various clinical isolates of *Neisseria gonorrhoeae*. Antimicrob. Agents Chemother. **12**:444–446.
8. **Maier, T. W., L. Zubrzycki, and M. B. Coyle.** 1975. Genetic analysis of drug resistance of *Neisseria gonorrhoeae*: identification and linkage relationships of loci controlling drug resistance. Antimicrob. Agents Chemother. **7**:676–681.
9. **Maier, T. W., L. Zubrzycki, M. B. Coyle, M. Chila, and P. Warner.** 1975. Genetic analysis of drug resistance in *Neisseria gonorrhoeae*: production of increased resistance by the combination of two antibiotic resistance loci. J. Bacteriol. **124**: 834–842.
10. **Maness, M. J., and P. F. Sparling.** 1973. Multiple antibiotic resistance due to a single mutation in *Neisseria gonorrhoeae*. J. Infect. Dis. **128**:321–330.
11. **Nordstorm, K., K. G. Ericksson-Greenberg, and H. G. Boman.** 1968. Resistance of *Escherichia coli* to penicillins. III. *AmpB*, a locus affecting episomally and chromosomally mediated resistance to ampicillin and chloramphenicol. Genet. Res. **12**: 157–168.
12. **Sarubbi, F. A., Jr., E. Blackman, and P. F. Sparling.** 1974. Genetic mapping of linked antibiotic resistance loci in *Neisseria gonorrhoeae*. J. Bacteriol. **120**:1284–1292.
13. **Shockley, T. E., and R. D. Hotchkiss.** 1970. Step-wise introduction of transformable penicillin resistance in *Pneumococcus*. Genetics **64**:397–408.
14. **Sparling, P. F.** 1966. Genetic transformation of *Neisseria gonorrhoeae* to streptomycin resistance. J. Bacteriol. **92**:1364–1371.
15. **Sparling, P. F., L. Guymon, and G. Biswas.** 1976. Antibiotic resistance in the gonococcus, p. 494–500. *In* D. Schlessinger (ed.), Microbiology—1976. American Society for Microbiology, Washington, D.C.
16. **Sparling, P. F., F. A. Sarubbi, Jr., and E. Blackman.** 1975. Inheritance of low-level resistance to penicillin, tetracycline, and chloramphenicol in *Neisseria gonorrhoeae*. J. Bacteriol. **124**:740–749.
17. **Spratt, S. K., F. Jones, T. E. Shockley, and J. H. Jackson.** 1980. Cotransformation of a serum resistance phenotype with genes for arginine biosynthesis in *Neisseria gonorrhoeae*. Infect. Immun. **29**:287–289.
18. **Warner, P. F., L. Zubrzycki, and M. Chila.** 1980. Polygenes and modifier genes for tetracycline and penicillin resistance in *Neisseria gonorrhoeae*. J. Gen. Microbiol. **117**:103–110.