JOURNAL OF BACTERIOLOGY, Nov. 1975, p. 750-756
Vol. 124, No. 2
Printed in U.S.A.
Copyright © 1975 American Society for Microbiology

Loss of Low-Level Antibiotic Resistance in *Neisseria gonorrhoeae* due to *env* Mutations

FELIX A. SARUBBI, JR., P. FREDERICK SPARLING,* ELEANOR BLACKMAN, AND EVELYN LEWIS

Departments of Medicine and Bacteriology and Immunology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514, and Department of Bacteriology, University of Bristol, Bristol, England*

Received for publication 5 May 1975

Mutations (*env*) which resulted in increased sensitivity of gonococci to diverse compounds were studied by transformation. Strains carrying an *env* mutation were more sensitive than wild-type strains to several antibiotics, dyes, and detergents. The *env* mutations resulted in complete phenotypic suppression of low-level resistance to these same drugs determined by mutation at *ery*. Recombination was observed in transformation crosses between various *env* mutants. The *env* locus was not linked to the cluster of antibiotic resistance genes near *str* and *spc*.

Maness and Sparling previously reported a class of mutant that resulted in pleiotropic loss of low-level resistance to multiple antibiotics in *Neisseria gonorrhoeae* strain FA5. Resistance to all drugs was restored by selection of back mutants resistant to any one (8). On this basis, it was postulated that one gene affected the response of FA5 to multiple drugs, and that many-fold increases in resistance to diverse drugs could develop by mutation at a single locus (8). Results of transformation experiments have shown, however, that low-level resistance of FA5 to these antibiotics was due to the sum of mutations at several independent loci (17). Two of these (*ery-1* and *penB1*) did result in nonspecific low-level resistance (17), but of a magnitude considerably smaller than observed previously during the single-step mutational loss and reacquisition of multiple drug resistance in FA5 (8).

Accordingly, we have reinvestigated the nature of the mutations in FA5 which resulted in loss of multiple drug resistance. These mutations, designated *env* because of their probable effect on the cell envelope, were shown to be phenotypic suppressors of low-level resistance determined by other loci. Mutations at two genetically distinct *env* sites resulted in similar Env phenotypes.

MATERIALS AND METHODS

All transformations and other methods have been described (13, 16, 17). In some instances, results of sensitivity testing are slightly different from those presented elsewhere for the same strains (17). These discrepancies are due to performance of experiments in different laboratories (Chapel Hill and Bristol) and probably reflect minor differences in quality of media, or perhaps the occasionally different sources of drugs used. (In Bristol, penicillin G was from Glaxo, erythromycin [Ery] was from Sigma, and tetracycline [Tet] was from Lederle). The discrepancies affected absolute levels of resistance, but not the relative magnitude of the changes due to the mutations under study.

Bacterial strains. Strains used are shown in Table 1. Wild-type strain FA18 was designated as strain 18 in an earlier paper (8); it was obtained as strain Ceylon 24 from A. Reyn. Strain FA32 was formerly designated 18 *strB10* (8). The *env* mutants FA32 and FA47 were both selected initially for high-level Str^R; the *env* mutations were therefore unselected and arose by chance as one of several other mutations induced by ultraviolet light. Strain FA52 was originally designated strain 5-2 (8). The genotypes of FA5 and FA48 have been fully described (13, 17). The genotypes of other strains were established in each instance by transformation to appropriate recipients, as described earlier (13, 17), or in the case of the *env* mutations as described in this paper.

The antibiotic phenotype of certain strains is designated in the text by brackets enclosing both the appropriate symbol and the minimum inhibitory concentration (MIC) in micrograms per milliliter for that drug. Thus, FA52 (Pen 0.12) indicates strain FA52 has an MIC for penicillin G of 0.12 μg/ml.

Introduction of env mutations. Two methods were used in attempts to introduce *env* mutations into various recipients. In the first, recipients were exposed to saturating (10 to 50 μg/ml) concentrations of transforming deoxyribonucleic acid (DNA) from an *env* donor, with subsequent scoring of unselected recipients for increased sensitivity to Ery by replica plating. In the second, recipients similarly exposed to *str env* DNA were incubated for 6 h at 36 C before streptomycin was added to the undersurface of the agar to give a final concentration of 300 μg/ml. After further incubation for 36 h at 36 C, *str* transform-

ants growing on the agar surface were directly replica-plated onto plates containing either slightly less Ery than required to inhibit growth of the recipients or no Ery. Colonies which failed to grow on plates with Ery were purified and carefully tested for sensitivity to many drugs.

RESULTS

Phenotype of env mutations. The *env-1* mutation in strain FA52 which was previously shown to result in partial or complete loss of low-level resistance of strain FA5 to penicillin (Pen), Tet, chloramphenicol (Chl), Ery, and rifampin (Rif) (8) also resulted in increased sensitivity to other antibiotics, dyes, and detergents (Table 2). Spontaneous back mutants of FA52 selected for resistance to one drug (FA56, a Tet^R mutant of FA52) were completely resistant to all drugs (Table 2). In comparison to FA19, which has been designated "wild type" since its

TABLE 1. *Strains of N. gonorrhoeae used*

Strain	Genotype	Origin
FA18	Wild type	A. Reyn (8)
FA32	*env-3 str-10 tet-3 chl-3 ery-3 penA3 penB3*	UV Str^R mutant of FA18 (8)
FA33	As FA32 but *env*$^+$	Spontaneous Pen^R mutant of FA32
FA5	*env*$^+$ *str-1 tet-1 chl-1 ery-1 penA1 penB1*	D. Kellogg (8)
FA52	As FA5 but *env-1*	UV mutant of FA5 (8)
FA56	As FA52 but *env*$^+$	Spontaneous Tet^R mutant of FA52
FA19	Wild type	A. Reyn (8)
FA47	*env-2 str-7 penA2*	UV Str^R mutant of FA19 (8)
FA48	As FA47 but *env*$^+$ *tet-2 chl-2 ery-2 penB2*	UV Pen^R mutant of FA47 (8)
FA102	*penA2*	Recombinant from FA48 × FA19 (17)
BR43	As FA102 but *str-10 env-3*	Recombinant from FA32 × FA102
FA140	*penA2 ery-2 penB2*	Multiple transformant from FA48 × FA19 (17)
BR54	As FA140 but *str-10 env-3*	Recombinant from FA32 × FA140
BR84	As FA140 but *str-1 env-1*	Recombinant from FA52 × FA140
BR87	As FA140 but *str-7 env-2*	Recombinant from FA47 × FA140
BR88	As FA47 but *rif-4*	Spontaneous mutant of FA47
BR89	As BR84 but *rif-4*	Recombinant from BR88 × BR84
BR90	As FA32 but *rif-4*	Recombinant from BR88 × FA32
FA164	As FA19 but *penA1 ery-1*	Two-step transformant from FA5 × FA19 (17)

TABLE 2. *Increased sensitivity to antibiotics, dyes, and detergents due to env mutations*

Strain	Description	MIC (μg/ml)[a]								
		Pen	Tet	Chl	Ery	Rif	Fus	Trx[b]	AO	CV
FA5	*env*$^+$ *str-1 tet-1 chl-1 ery-1 penA1 penB1*	2.0	4.0	8.0	4.0	0.5	1.0	>16.0	400	8.0
FA52	*env-1* mutant of FA5	0.12	1.0	1.0	0.06	0.03	0.015	0.06	100	1.0
FA56	*env*$^+$ mutant of FA52	2.0	4.0	8.0	4.0	0.5	1.0	>16.0	400	8.0
FA19	Wild type	0.007	0.25	0.5	0.25	0.12	0.12	0.5	100	2.0
FA102	*penA2*	0.06	0.25	0.5	0.25	0.12	0.12	0.5	100	4.0
BR43	*env-3* transformant of FA102	0.06	0.5	1.0	0.03	0.03	0.03	0.06	25	1.0
FA140	*penA2 ery-2 penB2*	1.0	1.0	1.0	1.0	0.5	1.0	>16.0	400	8.0
BR54	*env-3* transformant of FA140	0.06	0.25	0.25	0.03	0.03	0.03	0.06	50	1.0
BR84	*env-1* transformant of FA140	0.06	0.25	0.25	0.03	0.03	0.03	0.06	50	2.0
BR87	*env-2* transformant of FA140	0.06	0.12	0.25	0.06	0.03	0.03	0.12	50	1.0
Fold decrease due to *env* mutations:										
Compared to FA102					8	4	4	8	4	4
Compared to FA140		16	4	4	32	16	32	>256	8	8

[a] Abbreviations: Fus, fusidic acid; Trx, Triton X-100; AO, acridine orange; CV, crystal violet.
[b] MIC to Triton X-100 in milligrams per milliliter.

antibiotic sensitivities (8, 13) are typical of gonococci isolated in the pre-antibiotic era, FA52 was hypersensitive to many compounds, including Ery, Rif, fusidic acid (Fus), and Triton X-100 (Table 2).

Several other ultraviolet-induced mutants previously isolated from wild-type strains FA18 or FA19 (8), including *env-2* mutant FA47 and *env-3* mutant FA32, were essentially identical to FA52 in their hypersensitivity to Ery, Rif, Fus, and Triton X-100. Other genetic evidence presented below substantiated the similarity of FA32, FA47, and FA52; all contained mutations in loci designated *env*, which resulted in hypersensitivity to diverse compounds.

Phenotypic suppression of low-level resistance by env-1. FA5 has been shown to contain many mutations which additively resulted in low-level resistance to Pen, Ery, Tet, Chl, and other drugs (17). Since resistance to all was lost by an apparent single-site mutation in FA52 (8), we asked whether this mutation occurred in one of the genes for low-level resistance, or in a separate locus. Transforming DNA from *env-1* mutant FA52 was therefore introduced into wild-type strain FA19 or into *penA1 ery-1* strain FA164, with selection appropriate for each of the low-level resistance loci in FA5 (17). The results showed that the Ery-hypersensitive donor FA52 (Ery 0.06) was able to transfer resistance (Ery 4.0) to Ery-sensitive (Ery 0.25) recipient FA19, and the relatively Pen-sensitive (Pen 0.12) donor was able to transfer Pen^R (1.0) to recipient FA164 (Pen 0.25) (Table 3). By other criteria (17), these were typical *ery-1* and *penB1* transformants. Each of the other known loci in FA5 was also demonstrated in FA52 by transfer to FA19 (Table 3); the frequencies of co-transformation of linked markers and other features were similar to experiments using FA5 as donor (13, 17). We concluded that the mutation *env-1* which rendered FA5 antibiotic sensitive occurred at a locus separate from those for low-level resistance, and that it acted to phenotypically suppress the effects of some of them.

This conclusion was verified by other experiments. If the only difference between FA5 and FA52 were the presence of a mutation in FA52 (*env-1*), which phenotypically suppressed other loci for low-level resistance, then any env^+ strain should transform FA52 to antibiotic resistance. As predicted, DNA from wild-type-sensitive strain FA19 was able to transform the drug-hypersensitive mutant FA52 to antibiotic resistance. The transformants were all considerably more resistant to the selected drug than either the donor or the recipient (Table 4). Similar results were obtained with other wild-type donor strains (data not shown).

Moreover, the transformants of FA52 obtained from experiments with donor DNA from either sensitive or resistant strains were uniformly resistant to multiple drugs (Table 5). The results shown in Table 5 should be contrasted to similar experiments, which also used multiply resistant strain FA5 as donor but FA19 or other wild-type strains as recipient; under these conditions resistance to each drug was transferred independently (13). This difference is now easily understood; with wild-type recipients, selection was for each of several independent loci for antiobiotic resistance in FA5 (*penA1*, *ery-1*, and others) (13, 17), whereas with recipient FA52 selection with Pen, Ery, Chl, or Tet was actually for the env^+ marker in

TABLE 3. *Transfer by env-1 mutant FA52 of resistance to drugs to which it is phenotypically sensitive*[a]

Recipient	Selected phenotype (μg/ml)[b]	Donor markers selected	Transformation frequency (%)[c]	No. scored	Resistance to selected drug (MIC, μg/ml)		
					Donor	Recipient	Transformant
FA19	Pen^R (0.03)	*penA1*	0.03	15	0.12	0.007	0.06
	Str^R (300)	*str-1*	0.01	19	>8,000	20	>8,000
	Chl^R (0.5)	*chl-1*	0.006	19	1.0	0.5	2.0
	Tet^R (0.5)	*tet-1*	0.003	19	1.0	0.25	1.0
	Ery^R (0.5)	*ery-1*	0.002	19	0.06	0.25	2.0–4.0[d]
FA164	Pen^S (0.3)	*penB1*	0.001	19	0.12	0.25	0.5–1.0[d]

[a] Donor FA52: *penA1* // *ery-1* // *str-1* *tet-1* *chl-1* *penB1* // *env-1*
Recipients FA19 + + + + + + // +
FA164 *penA1* // *ery-1* // + + + + // +

[b] Numbers in parentheses give concentration of drug used in selection of transformants.

[c] Percentage of exposed cells transformed.

[d] Range of resistance found among transformants. All *penB1* transformants of FA164 were also two- to fourfold more Tet^R. All *penA1*, *str-1*, *chl-1*, and *tet-1* transformants were scored at a single phenotypic level of resistance.

FA5 (Table 5). The env^+ transformants all became multiply antibiotic resistant, because FA52 contained all the mutations which in aggregate produce this phenotype (Table 3) (17).

The experiments shown in Table 5 also established that the *env* locus in FA52 is not closely linked to the cluster of antibiotic resistance genes around *str* and *spc* (13), since no env^+ transformants (<2%) from FA50 (*spc-3* str^+ env^+) × FA52 (spc^+ *str-1 env-l*) acquired the donor's *spc* or *str* markers. Similarly, selecting for high-level Spc^R (*spc-3*) transformants did not result in any env^+ (low-level, multiply drug resistant) transformants (Table 5).

Map positions of tet-1 and chl-1. It was previously reported that the *str-1, tet-1,* and *chl-1* loci in FA5 were apparently arranged in the linear order *str-1, chl-1 tet-1*, in contrast to the more precisely mapped loci in FA48 which were in the order *str-7 tet-2 chl-2* (13). In the course of the present experiments, the map positions of these loci in FA5 were reinvestigated. A *str-1 tet-1 chl-1* transformant of FA19 (BR17) constructed by transformation from FA5 was used as source of transforming DNA. In the subsequent cross *str-1 tet-1 chl-1* × recipient FA19 (str^+ tet^+ chl^+), 33 of 129 *str-1* transformants were Tet^R (25.5%) and 21 of 129 were Chl^R (16.3%); there were 14 Tet^R Chl^S but only two Tet^S Chl^R recombinants. In addition, in the transformation cross *str-1 tet-1 chl-1* (donor) × str^+ *tet-2 chl-2* (recipient), none of 200 *str-1* transformants showed any increase or decrease in resistance to Tet or Chl. Therefore, the *tet-1* and *chl-1* in FA5 are in the same relative order as in FA48 (*str tet chl*); this revision is indicated in Tables 3 and 5.

TABLE 4. *Introduction of wild-type (sensitive) DNA into env-1 mutant FA52 results in antibiotic resistance*[a]

Selected phenotype (μg/ml)[b]	No. scored	Transformation frequency	Resistance to selected drug (MIC, μg/ml)		
			Donor	Recipient	Transformant
Pen^R (0.5)	19	0.01	0.007	0.12	1.0
Ery^R (0.5)	19	0.01	0.25	0.06	2.0–4.0[c]

[a] Donor, FA19; recipient, FA52. Donor DNA concentration limiting (0.01 μg/ml).

[b] Numbers in parentheses give concentration of drug used in selection of transformants.

[c] Range of resistance found among transformants.

Two classes of Env^+ transformants and mutants. Selection with low levels of Ery (0.12 to 0.25 μg/ml) in the experiments shown in Table 5 resulted in transformation frequencies (0.03 to 0.05%) slightly higher than observed with 0.50 μg of Ery per ml (transformation frequencies, 0.01 to 0.03%). Moreover, when selection was with 0.12 to 0.25 μg of Ery per ml, two classes of transformant were obtained; approximately 20% were fully resistant to Pen, Tet, Chl, and Ery, like FA5 (Table 2), whereas the remainder were only slightly more resistant to all these drugs. When selection was with 0.50 μg of Ery per ml, or with the levels of Pen, Tet, or Chl used in these experiments (Table 5), all transformants were fully resistant, like FA5.

Similar results were obtained when spontaneous resistant mutants were selected from FA52 (Table 6). Selection with very low-level Ery (0.10) resulted in two general classes of mutants: the majority were only partially Ery^R (MIC, 0.12 to 0.25; Table 6) and were generally

TABLE 5. *Transformation of env-1 mutant FA52 to multiple drug resistance by resistant or sensitive DNA*[a]

Donor	Selected phenotype (μg/ml)[b]	Transformation frequency	No. scored	% Acquiring resistance to unselected drug[c]					
				Pen^R	Ery^R	Tet^R	Chl^R	Spc^R	Str^S
FA5	Pen^R (0.25)	0.03	83	100	100	100	100	—[d]	—
	Ery^R (0.5)	0.03	192	100	100	100	100	—	—
	Tet^R (1.0)	0.01	19	100	100	100	100	—	—
	Chl^R (2.0)	0.02	38	100	100	100	100	—	—
FA50	Pen^R (0.50)	0.01	19	100	100	100	100	0	0
	Ery^R (0.25)	0.01	52	100	100	100	100	0	0
	Spc^R (200)	0.03	19	0	0	0	0	100	0

[a] Donors FA5 *penA1* // *ery-1* // *str-1 tet-1 chl-1* + *penB1* // +
FA50 + // + // + + + *spc-3* + // +
Recipient FA52 *penA1* // *ery-1* // *str-1 tet-1 chl-1* + *penB1* // *env-1*

[b] Numbers in parentheses give concentration of drug used in selection of transformants.

[c] When selection was with Pen, Tet, Chl, or 0.5 μg of Ery per ml, all transformants were phenotypically nearly identical to FA5 (MIC: Pen, 1.0 to 2.0; Tet, 2.0 to 4.0; Chl, 4.0 to 8.0; Ery, 2.0 to 4.0). When selection was with 0.25 μg of Ery per ml or less, two classes of transformant were obtained: 10 to 30% were phenotypically like FA5, and the remainder were two- to fourfold less resistant to all drugs than FA5.

[d] —, Not scored, as donor and recipient both were Spc^S Str^R.

TABLE 6. *Spontaneous Ery^R (env$^+$) mutants from env-1 strain FA52*[a]

Selected phenotype (μg/ml)[b]	Mutation frequency	No. scored	MIC of Ery (μg/ml)				
			0.12	0.25	0.50	1.0	2.0
Ery (0.1)	3.2×10^{-6}	64	11	34	1	4	14
Ery (0.5)	0.6×10^{-6}	10	0	0	0	0	10

[a] Phenotype of FA52 is Ery 0.06.

[b] Numbers in parentheses are concentration of drug used to select mutants.

correspondingly more resistant to other drugs (not shown). The minority were significantly more Ery^R (MIC, ≥ 1.0; Table 6) and were more resistant to other drugs also (not shown). Selection with higher levels of Ery (0.50) resulted in an approximate fivefold reduction in mutation frequency, but all mutants were fully resistant to Ery and other drugs and were thus similar phenotypically to the original resistant parent FA5.

There are several possible explanations for these results. The transformation results are compatible with the idea that the *env-1* mutation is actually a two-site mutation; transformation with donor DNA, which was wild type at both sites, might be expected to either partially or fully repair the defect in the *env* mutants, depending on whether one or both sites were repaired. If the two sites were closely linked, selection for repair of one site by use of very low concentrations of Ery would result in relatively frequent repair of both sites. On the other hand, the mutational results are not easily explained by a two-site model, since the fully resistant phenotype should only be restored by quite rare double mutations.

Introduction of env by transformation. No Ery-hypersensitive transformants of FA19 were observed among 2×10^4 unselected recipients of transforming DNA from *env-2* strain FA47, despite observed frequencies of 10^{-2} for *str-7* transformations in the same experiments. However, it was possible to construct isogenic *env* transformants of FA140 by first selecting for the donor *str* marker and then scoring for simultaneous introduction of the unlinked *env* mutation. Results of introduction of *env-1* from FA52 into FA140 by this technique are shown in Table 7. It was noteworthy that only 0.1% of *str-1* transformants were even moderately more sensitive to Ery and other drugs. The Ery-sensitive transformants were of two classes, in approximately equal numbers: one was partially sensitive to Ery (Ery 0.25) and other drugs, and the other fully sensitive (Ery 0.06). These results are also compatible with, but do not prove, that *env-1* is composed of mutations at two closely linked sites. One of the fully sensitive transformants was saved as BR84; its phenotype closely mimics the donor strain FA52 (Table 2).

Similar methods were used to construct the *env-2* and *env-3* transformants of FA140, BR87, and BR54, respectively (Table 2). The frequency of Str^R transformants which were also Ery^S was approximately 0.05% in both cases, and in each instance only fully sensitive (Ery 0.06) *env* transformants were obtained. An *env-3* transformant of FA102 was similarly constructed (BR43, Table 2), but no *env-1* transformants from FA52 × FA102 were observed despite scoring over 14,000 Str^R transformants for Ery^S.

Recombination between env mutants. It seemed possible that mutations at more than one site could result in the same "hypersensitive" phenotype. If this were so, transformation crosses between strains similar to FA52 but with mutations at different sites should result in *env*$^+$ (resistant) recombinants, whereas those with mutations at the same or adjacent sites should result in no or rare recombinants.

Preliminary results showed that resistant recombinants were obtained from crosses between *env-3* mutant FA32 and either *env-1* mutant FA52 or *env-2* mutant FA47, but not between FA47 and FA52 (not shown). This suggested that the *env-3* mutation occurred at a site distant from *env-1* and *env-2*. These results were confirmed in transformation crosses in which the isogenic *env* transformants were used as recipients, and derivatives of these strains or their parents which contained the *rif-4* locus for high-level Rif^R were used as donors (Table 8). Results showed that Ery^R (0.25) transformants were obtained with equal or greater frequency than Rif^R in crosses between *env-3* and either *env-1* or *env-2*, but were very rare to unobtainable in crosses between *env-1* and *env-*

TABLE 7. *Phenotype of Ery-sensitive transformants*[a] *from FA52 (env-1) × FA140 (env$^+$)*

No. of Ery^S transformants/no. scored	MIC (μg/ml)			
	Ery	Rif	Fus	Trx[b]
6/7736	0.25	0.12	0.12	0.50
3/7736[c]	0.06	0.03	0.03	0.06
Donor FA52	0.06	0.03	0.015	0.06
Recipient FA140	1.0	0.50	1.0	16.0

[a] Transformants were selected for donor marker *str-1* (Str 300), scored by replica plating for inability to grow on Ery 0.75, purified, and scored for sensitivity to all drugs.

[b] MIC to Triton X-100 (Trx) in milligrams per milliliter.

[c] BR84 (*env-1*) was one of the three fully sensitive transformants.

2. All Ery^R (0.25) transformants were also many-fold more resistant to Fus, Pen, and other drugs and were thus undoubtedly *env*$^+$. It therefore seemed clear that *env-3* occurred at a site distant from *env-1* and *env-2*, but that *env-1* and *env-2* were located in very close proximity to each other.

DISCUSSION

In these experiments we have studied mutations of the gonococcus which result in pleiotropic loss of low-level antibiotic resistance. These mutations resulted in increased sensitivity to antibiotics, dyes, and detergents and were thus similar to a variety of pleiotropic antibiotic-sensitive mutants which have been described in *Escherichia coli* (2-4, 9, 18, 19) and *Salmonella minnesota* (14, 15). In several of the *E. coli* (2, 3, 18, 19) and *Salmonella* (15) mutants, the outer envelope has been shown to be altered, resulting in increased permeability to various drugs. Additional evidence presented elsewhere (5) supports the idea that the envelope of our gonococcal antibiotic sensitivity mutants is altered, and hence the loci were designated *env*.

Previously, we showed that low-level resistance to most of these same compounds resulted from mutation at *ery* (17). In strains such as FA52, which carried both *ery-1* and *env-1*, the effects of *env-1* appeared completely dominant (Tables 2 and 3). Further evidence for phenotypic dominance of *env* over *ery* is provided by comparisons of the *env-3* transformants BR43 (*ery*$^+$) and BR54 (*ery-2*). Both are equally sensitive to Ery, Rif, Fus, Triton X-100 and acridine orange (Table 2). No clear explanation for this is possible yet, but if *ery* also affects cell envelope structure, as seems likely (5), then the *env* mutations must alter the envelope in a manner which effectively cancels the opposite effect due to *ery*. The *env* mutations also appeared to abolish the phenotypic effects of *penB*, as evidenced by loss of Pen^R and Tet^R in the *env* transformants of FA140 (Table 2). The *env* mutations had no effect on penicillin resistance determined by *penA* (compare *penA2 env*$^+$ strain FA102 and its *env-3* transformant BR43, Table 2) and also had no effect on high-level ribosomal (7) resistance to streptomycin or spectinomycin. It seems highly probable that the *env* mutations decrease the outer penetration barrier, resulting in increased sensitivity to diverse drugs, excepting instances where the target site is resistant to the drug.

Although the *env-1*, *env-2*, and *env-3* mutations produced essentially identical phenotypes, the recombination data (Table 8) clearly indicated that *env-3* is distant from *env-1* and *env-2*. The recombination index obtained in crosses between *env-3* and the others was always at least 1.0, which suggests the possibility that *env-3* occurs in a locus separate from *env-1* and *env-2*. This could also reflect high-frequency recombination between distant sites in a single locus, however, and in the absence of complementation analysis or knowledge of the gene products of the *env* mutations it is not possible to determine the number of loci involved.

Similar ambiguities remain in understanding the precise nature of the *env-1* mutation. Certain evidence suggested that *env-1* mutations might actually be a double mutation. In particular, two classes of Ery^R transformants were obtained in crosses between *env*$^+$ DNA and an *env-1* recipient, if selection was made with sufficiently low levels of Ery (Table 5), and two classes of Ery^S transformant were obtained when *env-1* DNA was introduced into an *env*$^+$ recipient (Table 7). Ultimate resolution of the genetic fine structure of the *env-1* mutation was not attempted.

TABLE 8. *Ery*^{R} *(env*$^+$*) recombinants in transformation between various env mutations*

Donor	Recipient	Transformants/ml[a]		Recombination index (Ery^R/Rif^R)
		Ery^R (0.25 μg/ml)[b]	Rif^R (5.0 μg/ml)	
BR90 *env-3 rif-4*	BR84 *env-1*	8.1×10^4	2.7×10^4	3.00
BR90 *env-3 rif-4*	BR87 *env-2*	5.4×10^4	2.4×10^4	2.25
BR90 *env-3 rif-4*	BR54 *env-3*	$<3 \times 10^0$	3.0×10^4	<0.0001
BR88 *env-2 rif-4*	BR84 *env-1*	$<3 \times 10^0$	1.1×10^3	<0.003
BR88 *env-2 rif-4*	BR87 *env-2*	$<3 \times 10^0$	2.6×10^3	<0.001
BR88 *env-2 rif-4*	BR54 *env-3*	1.8×10^5	1.5×10^5	1.20
BR89 *env-1 rif-4*	BR84 *env-1*	$<3 \times 10^0$	1.5×10^3	<0.002
BR89 *env-1 rif-4*	BR87 *env-2*	3×10^0	4.1×10^3	0.0007
BR89 *env-1 rif-4*	BR54 *env-3*	1.8×10^5	1.2×10^5	1.50

[a] Approximately 10^7 colony-forming units of the recipient per ml were exposed to 1.0 μg of donor DNA per ml.

[b] Numbers in parentheses are concentration of drug used to select transformants. All Ery^R transformants were as resistant as strain FA140 to many drugs (Rif, Fus, Ery, Pen) and were thus *env*$^+$. All Rif^R transformants were resistant to at least 500 μg of Rif per ml, but were not more resistant to other drugs. (Ten Ery^R and 10 Rif^R transformants were tested in each cross).

The *env* mutations are apparently relatively common among clinical gonococcal isolates. Based on the criteria of hypersensitivity to Ery, Rif, Fus, Triton X-100, and acridine orange as compared to strains FA19 and FA102, approximately 10% of 400 local isolates contained *env* mutations (unpublished data). Two of these have been studied genetically in preliminary experiments, and both are in every respect similar to FA52 (data not shown). The occurrence of both the pleiotropic drug hypersensitivity *env* mutants and the pleiotropic drug resistance *ery* and *penB* mutants among clinical isolates probably explains many of the observed correlations in drug resistance (1, 6, 8, 10–12). In addition, the relative frequency of the hypersensitive *env* mutants suggests a positive selective advantage other than drug resistance for such mutants in nature, although the basis for this is obscure. Growth rates of *env* mutants have not been faster than other gonococci in enriched broth cultures. The *env* mutants are probably at a selective disadvantage in vitro, due to their propensity to autolyze in plate culture or in broth (unpublished observations).

Finally, the previously published statement that mutation at a single locus may result in many-fold increases in resistance of the gonococcus to multiple clinically useful antibiotics (Pen, Ery, Tet, Chl) (8) needs revision. A single mutation may indeed have this effect, but probably only in *env* mutants like FA52, which contain many suppressed mutations to low-level resistance. Resistance to these drugs is initially acquired in many steps (17) but may be lost and then reacquired by mutations at one of the *env* loci.

ACKNOWLEDGMENTS

P.F.S. is indebted to Mark Richmond of the Department of Bacteriology, Bristol, where some of the experiments were performed.

This work was supported by Public Health Service grant A110646 from the National Institute of Allergy and Infectious Diseases, by an MRC grant to M. Richmond, and by Public Health Service Research Career Development Award A133032 To P.F.S. from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. Amies, C. R. 1969. Sensitivity of *Neisseria gonorrhoeae* to pencillin and other antibiotics. Br. J. Vener. Dis. 45:216–222.
2. Boman, H. G., K. Nordström, and S. Normark. 1974. Penicillin resistance in *Escherichia coli* K12: synergism between penicillinase and a barrier in the outer part of the envelope. Ann. N.Y. Acad. Sci. 235:569–585.
3. Ennis, H. L., and M. I. Bloomstein. 1974. Antibiotic-sensitive mutants of *Escherichia coli* possess altered outer membranes. Ann. N.Y. Acad. Sci. 235:593–600.
4. Foulds, J., and C. Barrett. 1973. Characterization of *Escherichia coli* mutants tolerant to bacteriocin JF246: two new classes of tolerant mutants. J. Bacteriol. 116:885–892.
5. Guymon, L. F., and P. F. Sparling. 1975. Altered crystal violet permeability and lytic behavior in antibiotic-resistant and -sensitive mutants of *Neisseria gonorrhoeae*. J. Bacteriol. 124:757–763.
6. Maier, T., H. R., Beilstein, and L. Zubrzycki. 1974. Multiple antibiotic resistance in *Neisseria gonorrhoeae*. Antimicrob. Agents Chemother. 6:22–28.
7. Maness, M., G. C. Foster, and P. F. Sparling. 1974. Ribosomal resistance to streptomycin and spectinomycin in *Neisseria gonorrhoeae*. J. Bacteriol. 120:1293–1299.
8. 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.
9. Normark, S., H. G. Boman, and E. Matsson. 1969. A mutant of *Escherichia coli* with anomalous cell division and ability to decrease episomally and chromosomally mediated resistance to ampicillin and several other antibiotics. J. Bacteriol. 97:1334–1342.
10. Phillips, I., D. Rimmer, M. Ridley, R. Lynn, and C. Warren. 1970. In-vitro activity of twelve antibacterial agents against *Neisseria gonorrhoeae*. Lancet 1:263–265.
11. Reyn, A., and M. W. Bentzon. 1968. A study of relationships between sensitivities of *Neisseria gonorrhoeae* to sodium penicillin G, four semisynthetic penicillins, spiramycin, and fusidic acid. Br. J. Vener. Dis. 44:140–150.
12. Reyn, A., and M. W. Bentzon. 1969. Relationships between the sensitivities in vitro of *Neisseria gonorrhoeae* to spiramycin, penicillin, streptomycin, tetracycline, and erythromycin. Br. J. Vener. Dis. 45:223–227.
13. 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.
14. Schlect, S., and O. Westphal. 1968. Antibiotica-Empfindlichkeit bei s- och R-formen von *Salmonella minnesota*. Naturwissenschaften 10:494–495.
15. Schlect, S., and O. Westphal. 1970. Untersuchungen zur Typisierung von *Salmonella* R-Formen. IV. Typisierung von *S. minnesota* R-Mutanten mittels Antibiotika. Zentralbl. Bacteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. 213:356–381.
16. Sparling, P. F. 1966. Genetic transformation of *Neisseria gonorrhoeae* to streptomycin resistance. J. Bacteriol. 92:1364–1371.
17. 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.
18. Tamaki, S., and M. Matsuhashi. 1973. Increase in sensitivity to antibiotics and lysozyme on deletion of lipopolysaccharides in *Escherichia coli* strains. J. Bacteriol. 114:453–454.
19. Tamaki, S., T. Sato, and M. Matsuhashi. 1971. Role of lipopolysaccharides in antibiotic resistance and bacteriophage adsorption of *Escherichia coli* K-12. J. Bacteriol. 105:968–975.