ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1984, p. 767–769
0066-4804/84/060767-03$02.00/0

Vol. 25, No. 6

Identification of an Envelope Mutation (*env-10*) Resulting in Increased Antibiotic Susceptibility and Pyocin Resistance in a Clinical Isolate of *Neisseria gonorrhoeae*

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A mutation (*env-10*) conferring increased susceptibility to drugs, dyes, and detergents was detected in a clinical isolate of *Neisseria gonorrhoeae*. In certain strains, *env-10* also affected susceptibility to pyocins. This mutation was phenotypically similar to but genotypically distinct from previously described *env* mutations.

Isogenic strains of *Neisseria gonorrhoeae* containing mutations which confer either increased resistance or susceptibility to dyes, detergents, and drugs have been studied extensively in this laboratory (1, 3, 4, 7). Using isogenic strains, Guymon et al. (3) demonstrated that the presence of an *mtr* (multiple transformable resistance) locus results in reduced outer membrane permeability and increased resistance to multiple antimicrobial agents. However, certain mutations, designated *env*, phenotypically reverse the effects of *mtr* (7) and result in increased outer membrane permeability and susceptibility to drugs, dyes, and detergents (3, 7). Two phenotypically identical but genetically distinct loci (*env-1* and *env-3*) independently suppress the effects of *mtr* (7). The *env-1* locus and the genetically indistinguishable *env-2* locus result in decreased peptidoglycan cross-linking (4).

Recently, we examined clinical isolates obtained from Denmark. One of us (I.L.) observed that one particularly resistant isolate (strain FA952) lost resistance to a variety of antimicrobial agents after several passages in vitro; a susceptible derivative of strain FA952 is designated strain FA953. We were curious to know whether loss of resistance was due to an *env*-like mutation. These and other gonococcal strains employed in this study are listed in Table 1.

We determined the MICs of a variety of dyes, detergents, and antibiotics for selected strains (Table 2). We also examined the susceptibility of these strains to pyocins (2, 6) and pooled normal human serum (8). Strain FA953 was remarkably susceptible to dyes, detergents, and antibiotics to which FA952 was relatively resistant; the phenotype of strain FA953 was similar to those of well-characterized isogenic *env-1*, *env-2*, and *env-3* strains BR54, BR84, and BR87. (7). Curiously, strains FA952 and FA953 differed from each other in susceptibility to lipopolysaccharide (LPS)-specific pyocins (2, 6): strain FA952 was susceptible to pyocins 1 and 103, whereas strain FA953 was resistant to the same pyocins.

Results from three experiments suggested that strain FA953 contained an *env* mutation responsible for hypersusceptibility to the antimicrobial agents listed in Table 2. First, spontaneous erythromycin-resistant (Ery^r) mutants of strain FA953 (e.g., strain FA968), which arose at a frequency of ca. 1.5×10^{-9}, exhibited levels of multiple antimicrobial resistance similar to that of strain FA952. Second, strain FA953 Ery^r transformants (e.g., strain FA970) obtained with sheared DNA from strain FA952 were also phenotypically identical to strain FA952. Third, transforming DNA from the antibiotic-susceptible strain FA953 was able to transfer a locus (*mtr-4*) for resistance to multiple antibiotics to strain FA19 (e.g., strain FA964). These results indicated that strain FA953 contained a phenotypically suppressed locus for multiple antibiotic resistance and that suppression of resistance probably was due to mutation at a single *env* locus.

To determine whether the *env* mutation present in strain FA953 was genotypically identical to *env-1*, *env-2*, or *env-3*, we sought to determine whether such loci would recombine;

TABLE 1. Strains of *Neisseria gonorrhoeae* employed

Strain	Relevant genotype	Origin or reference
FA952	*mtr-4 env-10*$^+$	Statens Seruminstitut, Copenhagen
FA953	*mtr-4 env-10*	Statens Seruminstitut, Copenhagen
FA963	As strain FA953, but *nal-4*	Strain FA953 spontaneous Nal^r
FA968	*mtr-4 env-10*$^+$	Strain FA953 spontaneous Ery^r
FA970	*mtr-4 env-10*$^+$	Strain FA953 Ery^r transformant[a]; strain FA952 × FA953
FA19	*mtr*$^+$ *env-10*$^+$	A. Reyn
FA171	As strain FA19, but *mtr-2 env-10*$^+$	3
FA964	*mtr-4 env-10*$^+$	Strain FA19 Ery^r transformant; strain FA952 × FA19
BR54	*mtr-2 env-3*	7
BR84	*mtr-2 env-1*	7
BR87	*mtr-2 env-2*	7
FA994	*mtr-4 env-10*	Strain FA964 Ery^s transformant[b]; strain FA953 × FA964

[a] Donor DNA was prepared as described previously (9) and used to transform competent gonococci as described by Sarubbi et al. (7). Ery^r transformants were selected with GCB agar overlays that contained sufficient erythromycin to afford a final drug concentration of 0.5 μg per ml.

[b] This Ery^s transformant was identified by initial selection for Str^r. Of 10,000 such Str^r transformants, only 1 was found to be Ery^s.

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TABLE 2. Susceptibility of gonococcal strains to antimicrobial agents

Strain	Susceptibility or MIC of:[a]									
	Pen	Ery	Str	Tet	Rif	TX	PB	CV	NHS	Pyocins 1 and 103
FA952	2	2	R	2	0.03	16	500	8	R	S
FA953	0.25	0.25	R	0.25	0.015	0.016	100	0.5	R	R
FA968	1	1	R	1	0.03	16	500	8	R	S
FA970	1	1	R	2	0.03	16	500	8	R	S
FA19	0.015	0.25	S	0.25	0.015	0.16	100	2	R	S
FA171	0.03	1	S	1	0.03	16	NT[b]	8	R	S
FA964	0.25	2	S	1	0.03	16	NT	8	R	S
FA994	0.06	0.03	R	0.12	0.03	0.016	NT	0.5	R	S
BR54[c]	0.06	0.03	R	0.25	0.03	0.06	NT	1	NT	S
BR84	0.06	0.03	R	0.25	0.03	0.06	NT	2	NT	S
BR87	0.06	0.06	R	0.12	0.03	0.12	NT	1	NT	S

[a] Abbreviations: Pen, penicillin G; Ery, erythromycin; Str, streptomycin; Tet, tetracycline; Rif, rifampin; TX, Triton-X; PB, polymyxin B; CV, crystal violet; NHS, normal human serum; R, resistant; S, susceptible. All values are expressed in micrograms per milliliter, except that TX is expressed in milligrams per milliliter.

[b] NT, Not tested.

[c] Values for strains BR54, BR84, and BR87 are from Sarubbi et al. (7).

high frequencies of recombination would indicate that such loci probably are distinct. Donor DNA prepared from a spontaneous nalidixic acid-resistant (Nal^r) mutant strain (FA963) of strain FA953 readily transformed the *env-1*, *env-2*, and *env-3* strains, but not strain FA953, to both Ery^r and Nal^r (Table 3). These data suggested that strain FA953 contained an *env* mutation heretofore undescribed; this mutation is termed *env-10*. We were unable to determine whether *env-10* recombined with the *env* loci (*env-4-9*) present in the clinical isolates studied by Eisenstein and Sparling (1) because the strains and donor DNA no longer were available.

Since pyocin resistance in gonococci is known to result from certain alterations of LPS structure (6, 8), we studied the chemistry of LPS in strains FA952, FA953, and FA19, as well as in an *mtr-4* derivative of strain of FA19 (strain FA964) and an *env-10* transformant of strain FA964 (strain FA994). Surprisingly, neither *mtr-4* nor *env-10* altered the susceptibility of strain FA19 to pyocins 1 and 103, whereas expression of *env-10* in strain FA953 resulted in pyocin resistance (Table 2). When either whole, solubilized gonococci or purified LPS (2) were electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the gel was stained by a silver staining protocol (2), LPS from strain FA952 migrated with a higher apparent molecular weight than did LPS from strains FA953, FA968 (FA953 spontaneous Ery^r), and FA970 (FA953 Ery^r transformant) (data not shown). However, neither *env-10* nor *mtr-4* altered the electrophoretic mobility of LPS when these mutations were introduced by transformation into strain FA19 (strains FA964 and FA994). Therefore, although the presence of *env-10* in strain FA953 affected pyocin susceptibility, it apparently did not alter the primary structure of LPS. Moreover, analysis of gas-liquid chromatography identified neither qualitative nor quantitative LPS differences attributable to either *mtr-4* and *env-10* (Shafer and Guymon, unpublished data).

TABLE 3. Demonstration that the *env* mutation in FA953 recombines with *env-1*, *env-2*, and *env-3*

Recipient strain (genotype)	Transformants per ml[a]		
	Ery^r	Nal^r	Recombination index[b]
BR54 (*env-3 nal*$^+$)	61,000	68,000	0.9
BR84 (*env-1 nal*$^+$)	260,000	110,000	2.4
BR87 (*env-2 nal*$^+$)	56,000	69,500	0.8
FA953 (*env-10 nal*$^+$)	112	120,000	0.0009

[a] Ery^r transformants were selected at 0.5 μg of erythromycin per ml, whereas Nal^r transformants were selected at 5 μg of nalidixic acid per ml.

[b] Recombination index is defined as Ery^r transformants per milliliter/Nal^r transformants per milliliter.

Previous studies by Guymon et al. (3) demonstrated that an isogenic *mtr-2* derivative of strain FA19 (strain FA171) contained increased amounts of a 52,000-molecular-weight (52K) outer membrane protein; introduction of *env-1* or *env-2* (but not *env-3*) reduced the amounts of this protein. To determine whether either *mtr-4* or *env-10* affected the amount of this 52K protein, Sarkosyl-extracted outer membranes (4) prepared from strains FA952, FA953, FA968, and FA970 were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4). The amount of the 52K protein was greatly reduced in strain FA953 (*mtr-4 env-10*) outer membranes as compared with outer membranes of strain FA952 (*mtr-4 env-10*$^+$). Ery^r (*env-10*$^+$) mutants or transformants of strain FA953 (strains FA968 and FA970) contained quantities of the 52K protein similar to those in strain FA952 (data not shown). Thus, although *env-10* recombined with high frequency with both *env-1* and *env-2* and therefore may produce a different gene product, it resulted in alterations of the chemistry of the outer membrane similar to those effected by *env-1* and *env-2*. The mechanism by which *env-10*, *env-1*, or *env-2* alters the chemistry of the outer membrane is unknown. It is also unknown why *env-10* alters susceptibility to pyocins in strain FA952 derivatives, but not in strain FA19 derivatives.

It is unclear why clinical isolates of the gonococcus should contain *env* mutations, since these mutations increase susceptibility to antibiotics and other drugs. It is easier to understand the occurrence of mutations to nonspecific resistance such as *mtr*; these confer resistance not only to antibiotics, but also to bile salts and fatty acids, which may explain the frequency with which strains containing *mtr* mutations are isolated from rectal cultures (5). The apparent frequency of genetically distinct *env* mutations in clinical isolates from various parts of the world and the nearly uniform presence of phenotypically suppressed *mtr* mutations in isolates containing *env* mutations (as in strain

FA953) suggest that *mtr* mutations are selected first by antibiotics or other toxic substances such as fatty acids. Subsequent mutations at any of several genetically distinct *env* loci must result in biological advantage, even though antibiotic resistance is decreased.

We thank E. Blackman for excellent technical assistance, J. Mueller and L. Brooks for typing, and J. R. Scott for critical review of this manuscript.

This work was supported by National Research Service award AI06369 (W.M.S.) and by Public Health Service award AI15036 (P.F.S. and L.F.G.), both from the National Institute of Allergy and Infectious Diseases.

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