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Recombination Near the Antibiotic Resistance Locus *penB* Results in Antigenic Variation of Gonococcal Outer Membrane Protein I

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In gonococci, the nonspecific antimicrobial resistance locus *penB* is known to be closely linked to loci designated *nmp* that alter the M_r and antigenicity of the outer membrane porin protein I (P.I). We report that after selection for the linked donor *penB* locus, occasional recombinants expressed P.I with some epitopes from each parent. These hybrid P.I antigens were stable on subculture and were transformed at a locus closely linked to *penB*. The hybrid P.I antigens were detected with monoclonal antibodies in both coagglutination and Western blot assays. The alterations of P.I antigenicity may have resulted from recombination between structural genes for P.I that are closely linked to *penB*.

Serological classification of *Neisseria gonorrhoeae* is based at present on epitopes of the principal outer membrane protein (P.I). In the original scheme, multiple serogroups were delineated, of which the most common were serogroups WI and WII/WIII (17). Analysis of peptide maps suggests that P.Is from serogroups WI and WII/WIII are distinctly different proteins (16), although each is a porin (F. Greco, M. Blake, E. Gotschlich, and A. Mauro, Fed. Proc. **39**:1813, 1980). Serogroup WI is now usually designated P.IA, and WII/WIII is designated P.IB (16); each can be further divided into multiple serovars on the basis of reactions with a panel of monoclonal antibodies (9). Unlike gonococcal pili or outer membrane protein II, which are genetically highly unstable (5, 15), P.I does not undergo spontaneous antigenic variation at high frequency.

Genetic loci designated *nmp* that affect the M_r and antigenicity of gonococcal P.I are very closely linked to *sac* (serum resistance) and *penB* (low-level nonspecific antibiotic resistance) loci and less closely to genes determining resistance to streptomycin (*str*) or spectinomycin (*spc*) (5). The locus designated *nmp-1* results in the replacement of the recipient P.I by the donor 39,000-molecular-weight P.I (3); the serovar of the *nmp-1*-determined protein is P.IB-1 (unpublished data). The locus designated *nmp-3* results in the replacement of the recipient P.I by the donor 39,000-molecular-weight P.I, serovar P.IB-9 (19; unpublished data). The *nmp-2* locus results in the replacement of the recipient P.I by the donor 37,200-molecular-weight P.I (4). The *nmp-4* locus determines a 36,500-molecular-weight serovar P.IA-1 species of P.I (unpublished data). It is not known whether the *nmp* loci are structural genes for P.I; they could be regulatory genes that alter the expression of the structural genes, or they could be modifiers of the products of the structural genes (3).

In this report we demonstrate that a small proportion of recombinants selected for the closely linked donor *penB* locus acquire a novel species of P.I with certain epitopes from each parent. The results have important implications for understanding the genetics of P.I expression and possibly for the mechanisms of variation of P.I in nature.

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MATERIALS AND METHODS

The gonococcal strains used in this study are listed in Table 1. Growth conditions and media were as previously described (18). DNA was prepared by the method of Marmur (14). Transformation experiments with piliated recipients were performed by previously described methods (18), with limiting concentrations of DNA. Transformants for *penA4* were selected with 0.03 μg of penicillin G per ml, transformants for *mtr-4* were selected with 0.5 μg of erythromycin per ml, and transformants for *penB5* were selected with 0.37 μg of penicillin G per ml. The MICs for transformants were determined for penicillin G, tetracycline, and erythromycin by the agar plate dilution technique (20). Serotyping by coagglutination tests was performed with six monoclonal antibodies against P.IA (WI-serogroup specific) and six monoclonal antibodies against P.IB (WII/WIII-serogroup specific) as described previously (21). The monoclonal antibodies against P.IA were 4A12, 4G5, 2F12, 6D9, 5G9, and 5D1, and those against P.IB were 3C8, 1F5, 2D6, 2G2, 2D4, and 2H1. These reagents, already prepared for use in serotyping, were obtained by courtesy of the Centers for Disease Control, Atlanta, Ga.

Outer membrane preparations were made in lithium acetate buffer by the method of Lambden and Heckels (11). P.I was separated from other proteins by electrophoresis of samples containing approximately 30 μg of protein on sodium dodecyl sulfate–10% polyacrylamide gels in a Laemmli buffer system (10). Western blotting was performed by the method of Burnette (1). The proteins were transferred from the gel to nitrocellulose with 0.1% Zwittergent detergent 3-14 (Calbiochem-Behring) added to the transfer buffer (12). A 1:500 dilution of ascites fluid containing monoclonal antibodies against P.I was allowed to bind to proteins on the nitrocellulose following blocking of nitrocellulose with a 5% bovine serum albumin solution. Monoclonal antibodies in ascites were generously supplied by M. Tam (Genetic Systems). Radioiodinated staphylococcal protein A (specific activity, 1 μCi/ml; Amersham Corp.) was added for radiographic detection of bound antibody. The nitrocellulose was exposed at −70°C to Kodak XR film with an intensifying screen for 48 to 72 h.

One-dimensional peptide maps of P.I were generated by

TABLE 1. *N. gonorrhoeae* strains

Strain	Relevant genotype	Protein I serovar	MIC of penicillin G (μg/ml)	Source or reference
FA19	Wild type, *nmp-4*	IA-1	0.007	13
FA6140	*penA4 mtr-4 penB5 nmp-5*	IB-1	4.0	Local isolate
FA6142	*penA4 mtr-4 nmp-4*	IA-1	0.25	Transformant, FA6140 × FA19
FA6143	*penA4 mtr-4 penB5 nmp-5*	IB-1	0.5	Transformant, FA6140 × FA6142
FA6149	*penA4 mtr-4 penB5 nmp-6*	IA/IB	0.5	Transformant, FA6140 × FA6142
F62	wild type, *nmp-2*	IB-7	0.007	13
FA6212	*penA4 mtr-4 nmp-2*	IB-7	0.25	Transformant, FA6140 × F62
FA6213	*penA4 mtr-4 penB5 nmp-5*	IB-1	1.0	Transformant, FA6140 × FA6212
FA6214	*penA4 mtr-4 penB5 nmp-7*	IB-2	1.0	Transformant, FA6140 × FA6212
FA6215	*penA4 mtr-4 penB5 nmp-8*	IB-5	1.0	Transformant, FA6140 × FA6212
FA6216	*penA4 mtr-4 penB5 nmp-2*	IB-7	1.0	Transformant, FA6140 × FA6212

the limited proteolysis technique of Cleveland et al. (6). Bands corresponding to P.I were excised from sodium dodecyl sulfate–10% polyacrylamide gels and then digested for 30 min with 1 μg of either *Staphylococcus aureus* V8 protease (Sigma Chemical Co.), chymotrypsin (Calbiochem-Behring), or trypsin (Calbiochem-Behring) per ml. Peptide maps were examined after being silver stained by the technique of Wray et al. (22).

RESULTS

An initial impetus for our experiments was interest in the genetic basis of relatively high-level penicillin resistance (penicillin G MIC, 2 to 4 μg/ml) in beta-lactamase-negative gonococci (7). DNA from one such Pen^r strain, FA6140 (P.IB-1), was prepared and used in a series of transformations into Pen^s FA19 (P.IA-1) to increase its level of antibiotic resistance. Results demonstrated that Pen^r was due in part to multiple chromosomal mutations very similar to those described previously for somewhat less resistant isolates (5, 20). FA6140 was shown to contain mutations designated *penA4* (penicillin resistance), *mtr-4* (nonspecific multiple drug resistance), and *penB5* (nonspecific resistance). Details of these experiments will be published elsewhere.

Coagglutination assays of whole cells revealed no change in recipient P.I serovar in 30 *penA4* transformants or 30 *mtr-4* transformants. When the donor *penB5* locus was introduced from FA6140 into a *penA4 mtr-4* transformant of FA19 (FA6142), the P.I serovar of the recipient changed in each of 120 transformants characterized in three separate transformations (Table 2). Of these, 119 acquired the P.IB-1 serovar pattern of the donor; FA6143 is representative. The locus for the expression of P.IB-1 was designated *nmp-5*. One transformant (FA6149) acquired an apparently hybrid P.I with some donor P.IB epitopes and some recipient P.IA epitopes; other donor and recipient epitopes were lost in FA6149. Single-colony isolates of FA6149 all showed stable expression of the new serovar, apparently excluding the possibility of a mixed culture containing some cells with P.IA and others with P.IB. When transforming DNA from FA6149 was introduced into FA6142, each of 20 tested *penB5* (Pen^r) transformants acquired a serovar identical to that of FA6149. The locus determining the expression of the P.IA/P.IB protein was designated *nmp-6*.

When FA6140 (P.IB-1) was introduced into a recipient with a different P.IB serovar (F62; P.IB-7), similar results were obtained. No change in recipient P.IB serovar was seen by coagglutination with the introduction of either *penA4* (21 transformants tested) or *mtr-4* (17 transformants tested). When *penB5* was introduced from FA6140 into the *penA4 mtr-4* derivative of F62 (FA6212), a concomitant serovar change occurred in most transformants (Table 3). The most common class (86.8%) acquired the donor P.IB-1 serovar; FA6213 is representative of these. A few transformants (8 of 114) such as FA6216 retained the recipient P.IB-7 serovar. Interestingly, two classes of novel P.IB transformants were noted (FA6214 and FA6215). The serovars of FA6214 (P.IB-2) and FA6215 (P.IB-5) were stable during 3 months of serial passage, and multiple single-colony isolates of each gave identical serovar patterns. When transforming DNA from FA6214 (P.IB-2) was introduced into FA6212 (P.IB-7), 23 of 28 *penB5* (Pen^r) transformants acquired the donor P.IB-2 serovar; the remainder retained the serovar of the recipient. Introduction of FA6215 (P.IB-5) DNA into FA6212 resulted in 12 of 24 *penB5* transformants with the donor P.IB-5 serovar; the remainder retained the serovar of the recipient. Loci for the expression of P.IB-2 and P.IB-5 were designated *nmp-7* and *nmp-8*, respectively.

TABLE 2. Transformation of P.I serovar specificity and appearance of a novel serovar in crosses between a P.IB donor and P.IA recipient

Strain	Reactions with monoclonal antibodies against:												Serovar	% Transformants (120 tested)
	P.IA						P.IB							
	4A12	4G5	2F12	6D9	5G9	5D1	3C8	1F5	2D6	2G2	2D4	2H1		
Donor; FA6140	−	−	−	−	−	−	+	+	+	−	−	+	IB-1	
Recipient; FA6142	+	+	+	+	+	+	−	−	−	−	−	−	IA-1	
Transformant[a]; FA6149	−	+	+	−	−	−	+	−	+	−	−	+	IA/IB	0.8
Transformant; FA6143	−	−	−	−	−	−	+	+	+	−	−	+	IB-1	99.2

[a] Selection was made for the closely linked donor *penB* locus on plates containing 0.37 μg of penicillin G per ml. Pen^r transformants were scored by coagglutination reactions for the P.I serovars. The linear order of the P.I epitopes reactive with the monoclonal antibodies is unknown, and the listed order of monoclonal antibodies bears no necessary relationship to the linear structures of P.I in the various strains.

Since the coagglutination reactions involved the use of whole boiled bacterial cells, it seemed possible that the altered patterns of reactivity with P.I-specific monoclonal antibodies were not due to a change in P.I, but rather to a change in the surrounding cell matrix. Accordingly, the Western blot technique was used to test reactions of isolated P.I with the monoclonal antibodies. Outer membrane preparations were made of the donor FA6140, the recipient FA6142, and the P.IA/P.IB hybrid transformant, FA6149. P.I was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and allowed to react with P.IB- or P.IA-specific monoclonal antibodies. Of the six P.IB-specific monoclonal antibodies tested, only 1F5 gave a positive signal by Western blot. Monoclonal 1F5 reacted with the donor FA6140 P.IB, but not with FA6142 or FA6149 P.I, consistent with the coagglutination results (data not shown). Of the P.IA-specific antibodies, 4A12 and 5D1 were poorly or not reactive by Western blot against the P.IA recipient (FA6142) and were not tested further. Antibodies 6D9 and 5G9, which reacted in coagglutination tests with FA6142 (P.IA) but not FA6149 (P.IA/P.IB hybrid), gave identical results by Western blot (Fig. 1). Monoclonal antibody 4G5, which reacted with both recipient FA6142 and recombinant FA6149 by coagglutination, also reacted in immunoblots with the P.I of each of these strains, but not with the donor FA6140 P.IB. These results indicated that the P.I protein of the P.IA/P.IB hybrid FA6149 was indeed structurally altered, having lost two of the three epitopes detected by these antibodies, while retaining the third. The result with monoclonal antibody 2F12 was more complicated, since membranes of the P.IB donor FA6140 contained a band of lower M_r than P.IB that bound 2F12; this is in contrast to the coagglutination results, in which FA6140 did not react with this P.IA-specific monoclonal antibody. The P.IA recipient FA6142 and the P.IA/P.IB hybrid (FA6149) both showed P.I binding of this antibody, although 2F12 bound to the P.IA/P.IB hybrid protein relatively weakly. This may indicate that the structural alterations in the FA6149 P.I lowered the affinity of the 2F12-epitope for its antibody. Small amounts of lower-molecular-weight material binding 2F12 was also seen in both FA6142 and FA6149 membranes. The relationship of these 2F12-binding proteins to P.IA is unclear at present.

Similar experiments were undertaken with the novel P.IB recombinants that resulted from the cross between donor FA6140 (P.IB-1) and recipient FA6212 (P.IB-7). Again, the only P.IB-specific monoclonal antibody that reacted in Western blots was 1F5. The results (Fig. 2) were identical with those found by coagglutination: donor strain FA6140 and recombinants FA6213 and FA6215 reacted with monoclonal antibody 1F5 in immunoblots, but FA6214 and recipient FA6212 did not. The immunoblotting results indicated that the different reactions with monoclonal antibodies in coagglutination reactions probably were not due to varied exposure of common P.I epitopes on the surface of the transformed cells, but rather to intrinsic differences in P.I epitopes in the various transformants.

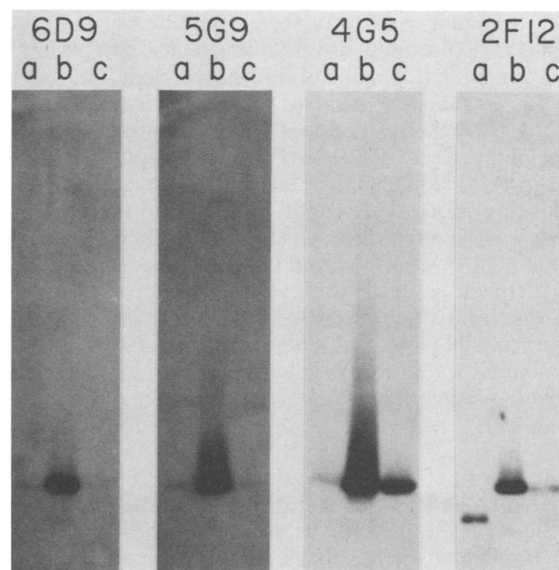

FIG. 1. Immunoblotting of gonococcal outer membrane proteins with P.IA-specific monoclonal antibodies. The monoclonal antibody used to probe each blot is designated at the top of the figure. For each panel, the membranes were isolated from the following strains: lane a, FA6140 (P.IB donor); lane b, FA6142 (P.IA recipient); lane c, transformant FA6149 (P.IA/P.IB hybrid).

TABLE 3. Transformation of P.I serovar specificity and the appearance of new serovars in crosses between parents differing in P.IB serovar

Strain	Reactions with monoclonal antibodies against P.IB						Serovar	% Transformants (114 tested)
	3C8	1F5	2D6	2G2	2D4	2H1		
Donor; FA6140	+	+	+	−	−	+	IB-1	
Recipient; FA6212	−	−	+	+	−	+	IB-7	
Transformant[a]; FA6213	+	+	+	−	−	+	IB-1	86.8
Transformant; FA6214	+	−	+	−	−	+	IB-2	1.8
Transformant; FA6215	−	+	+	+	−	+	IB-5	4.4
Transformant; FA6216	−	−	+	+	−	+	IB-7	7.0

[a] Selection was made for the closely linked donor *penB* locus on plates containing 0.37 μg of penicillin G per ml. Pen^r transformants were scored by coagglutination reactions for their P.I serovars. All were unreactive with six tested monoclonal antibodies with specificity for P.IA.

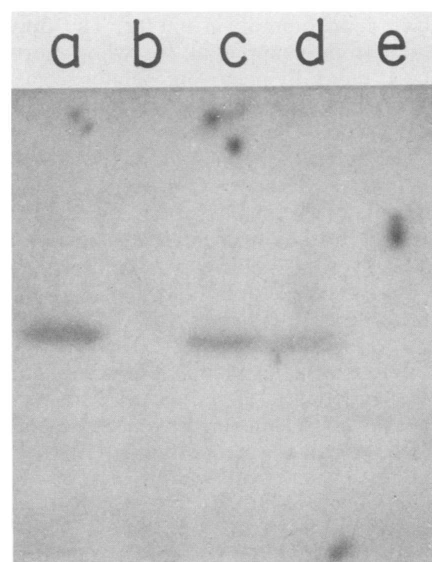

FIG. 2. Immunoblotting of gonococcal outer membrane proteins with P.IB-specific monoclonal antibody 1F5. Donor strain FA6140 is in lane a. Recipient strain FA6212 is in lane b. Transformants FA6213, FA6215, and FA6214 are in lanes c through e, respectively.

We attempted to identify structural differences in recombinant P.I expressing novel epitopes by comparing one-dimensional peptide maps of donor, recipient, and recombinant P.I. For this experiment, we chose recombinant strain FA6149 (P.IA/P.IB), its donor parent FA6140 (P.IB-1), and its recipient parent FA6142 (P.IA-1), reasoning that differences in peptide maps might be most obvious in a hybrid P.I expressing both P.IA and P.IB epitopes. The one-dimensional limited proteolysis peptide map of P.I from recombinant FA6149 was indistinguishable from that of P.I from recipient parent FA6142; both were obviously different from P.I from donor parent FA6140 (results not shown). Hence, possible chemical differences in P.I structure in the recombinant were too subtle to be detected by this relatively insensitive technique.

DISCUSSION

Cotransformation of a locus affecting P.I with the introduction of *penB* is not a new observation. According to Cannon et al. (3), it would be expected in 98% of transformants. Bygdeman et al. also found 100% cotransformation between a locus for low-level nonspecific antimicrobial resistance and P.I serogroup specificity (2). It was therefore not surprising that the vast majority of *penB5* transformants of FA6142 (P.IA-1) and FA6212 (P.IB-7) acquired a serovar pattern identical to that of donor FA6140 (P.IB-1). Of the remaining transformants, a few retained the serovar of the recipient. The cotransformation frequency between *penB5* and *nmp-5* locus affecting P.I expression was much higher when FA6142 rather than FA6212 was the recipient; the reasons for this are not clear. Although the present experiments actually never separated the *penB5* (Pen^r) and *nmp-5* (P.I) loci when FA6142 was the recipient, previous experiments involving the use of sheared transforming DNA containing the *nmp-1* locus and a recipient strain closely related to FA6142 apparently did so, based on the M_r of P.I (3).

Some transformants acquired unique serovar patterns, including reactive epitopes from both parents. The new P.I serovars were seen both in crosses between strains with different antigenic forms of P.IB (7 of 114) and in crosses of P.IB and P.IA strains (1 of 120). The P.IB/P.IA hybrid is apparently very uncommon in nature. The only report of such a strain is from Knapp et al. (9), who reported seeing 2 strains of 1,433 naturally occurring isolates from 12 different countries which reacted with one P.IB reagent (1F5) and one P.IA reagent (4G5). The reactivity with 4G5 was lost on subculture, and peptide mapping demonstrated that the P.I molecule belonged to the P.IB type (J. Knapp, personal communication). The P.IA/P.IB hybrid FA6149 found as a recombinant in this study was unlike that reported by Knapp et al. in that it was stable on subculture and showed reactivity of two P.IA epitopes (4G5 and 2F12) in addition to reactivity of three P.IB epitopes (3C8, 2D6, and 2H1).

Recently, we identified another P.IA/P.IB hybrid among transformants constructed in this laboratory during earlier studies of the genetics of gonococcal serum resistance (4). Using a *str-7* P.IB-7 serum sensitive strain FA610 as donor and str^+ P.IA-1 serum-resistant strain FA19 as recipient, we selected a *str-7* serum-sensitive transformant (FA628) that acquired a unique serovar pattern with reactivity to four P.IA epitopes (4A12, 4G5, 2F12, 6D9) and one P.IB epitope (2D6) (19; unpublished data). The formation of donor-recipient P.I hybrids was not appreciated previously because of the lack of the P.I monoclonal antibodies for serotyping. Earlier studies that involved the use of migration of P.I in sodium dodecyl sulfate-polyacrylamide gel electrophoresis for identification of donor or recipient P.I concluded that the *nmp* and *sac* (serum sensitivity) loci were closely linked but separable (4). The present result does not invalidate the former conclusion, but further analysis of the genetics of serum sensitivity and P.I structure by using P.I monoclonal antibodies appears warranted.

The occurrence of apparent P.I recombinants such as FA6149, FA6214, and FA6215 with epitopes from both the donor and the recipient strengthens but does not prove the hypothesis that the *nmp* loci are actually P.I structural gene(s). If structural genes for P.I (e.g., *nmp*) were closely linked to *penB*, selection for *penB* might be expected to result occasionally in intragenic recombinants of the *nmp* loci. Our results are consistent with the hypothesis that structural genes for P.IA and P.IB are closely linked to each other and to the *penB* locus. However, more work must be done to firmly answer these questions, including more complete analysis of the chemical structures of the parental and hybrid P.I molecules and molecular cloning and sequencing of DNA in the *nmp-penB* region.

It is possible that the close linkage between loci such as *penB5* and the *nmp* loci affecting P.I antigenicity contributes indirectly to the variation of P.I in nature. Selection by antibiotic pressures for in vivo *penB* transformants might occasionally give rise to novel P.I recombinants of the type observed in this report. There is reason to believe that gonococci undergo transformation in vivo (5, 8).

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