JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1977, p. 244-248

Vol. 6, No. 3
Printed in U.S.A.

Rapid Method for Auxotyping Multiple Strains of *Neisseria gonorrhoeae*

HELEN B. SHORT, VICKI B. PLOSCOWE, JEROME A. WEISS, AND FRANK E. YOUNG*

Department of Microbiology, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

Received for publication 15 April 1977

A rapid method for auxotyping strains was developed that uses microtiter plates. This miniplate technique enables rapid identification of major auxotypes present in clinical strains. Additional growth requirements can be identified by adding individual amino acid supplements to complete gonococcal genetic medium. Analysis of 8 clones from 40 patients revealed that 10 had more than 1 auxotype. Deoxyribonucleic acid-mediated transformation can be used to establish whether the strains with apparently more than one auxotype are defective in the same locus in each of the involved biosynthetic pathways. Selection of more than one clone is required in precise epidemiological studies.

The use of auxotyping to classify *Neisseria gonorrhoeae* for epidemiological studies was described previously (3). A major deterrent to the adoption of auxotyping in the clinical laboratory has been the laborious preparation and expense of complex chemically defined medium. Nevertheless, certain phenotypes of *N. gonorrhoeae* may have a greater pathogenicity and are implicated more frequently in disseminated gonococcal infection (9). Most studies of auxotyping have determined phenotypic patterns by selecting a single colony from the specimen. By this procedure, it has been impossible to determine whether infection may occur with more than one strain. This communication describes results of the auxotyping of multiple clones from a clinical specimen and introduces a rapid, economical means, using microtiter plates, to auxotype a large number of strains. The use of microtiter plates precludes cross-feeding that may occur between strains (3, 10). By examining a number of clones from the patient's culture for auxotrophic requirements and further classifying that auxotype by performing rapid deoxyribonucleic acid (DNA)-mediated transformation analyses, epidemiological studies can be readily carried out.

MATERIALS AND METHODS

Bacterial strains. Multiple clones of *N. gonorrhoeae*, isolated from cervical specimens of 40 patients seen at the Monroe County Venereal Disease Clinic, were studied. Strain F62 was obtained from D. Kellogg (Center for Disease Control, Atlanta, Ga.) and strain H4 from F. Tyeryar (National Institutes of Health, Bethesda, Md.). The specimens were isolated on Thayer-Martin agar and identified as *N. gonorrhoeae* by the Diagnostic Microbiology Laboratory at Strong Memorial Hospital. Eight well-isolated colonies on Thayer-Martin agar were selected for study on each patient. These clones were subcultured to GC medium base (Difco Laboratories, Detroit, Mich.) supplemented with IsoVitaleX (GCI; Bioquest, Div. of Becton, Dickinson & Co., Cockeysville, Md.) and were further purified by two successive single-colony selections. Auxotyping, sugar utilization studies, oxidase reaction, and Gram stain were performed.

Medium. Gonococcal genetic medium (GGM) was prepared as described by LaScolea and Young (10). Because some strains were stimulated by L-valine, the medium was modified to include 125 μg of L-valine per ml. Phenotyping was performed on the basis of the following amino acid supplements: L-cysteine/L-cystine, L-proline, L-arginine, L-methionine, L-serine, L-isoleucine, L-valine, and L-glutamic acid. Auxotyping could be readily accomplished by deleting an amino acid sequentially or completely with the exception of L-cysteine/L-cystine, which is required for growth by all *N. gonorrhoeae* (4).

Samples (0.2 ml) of the complete medium and the medium minus each amino acid in 1% Oxoid purified agar (Oxoid Ltd., London) were dispensed into microtiter plate wells (Falcon Plastics, Oxnard, Calif.). Each vertical row contained a single medium. The 12 vertical rows in the microtiter plate were used as follows: (i) complete; (ii) complete minus L-cysteine/L-cystine; (iii) complete minus L-valine; (iv) complete minus L-proline; (v) complete minus L-glutamic acid; (vi) complete minus L-arginine; (vii) complete minus L-methionine; (viii) complete minus L-serine; and (ix) complete minus L-isoleucine. Rows 10 and 11 contained cysteine-Trypicase agar (CTA) for sugar utilization studies. Row 10 contained CTA-1% glucose, and row 11 was CTA-1% lactose-1% sucrose-1% maltose, combined in equal parts. Row 12, GCI, was used as a growth control, as well as for testing oxidase reaction and Gram stain.

One filled microtiter plate (designated "miniplate") provided the necessary amount of medium to perform eight complete auxotypings, sugar utilization studies, and biochemical and microscopic confirmation of the strain tested, as shown in Fig. 1.

Strains that failed to grow on modified GGM were further tested for additional amino acid requirements. Modified GGM was prepared, and 40 ml was dispensed into 150-mm petri dishes. For most analyses, sterile paper disks (Baltimore Biological Laboratory, Cockeysville, Md.) were saturated with 20 μl of a 10-mg/ml stock solution of the following amino acids: L-lysine, L-tyrosine, L-tryptophan, L-leucine, L-threonine, L-alanine, L-glycine, L-phenylalanine, and L-histidine. In other experiments, diaminopimelic acid, glucosamine, and L-glutamine were added to this series. A suspension of the organism to be tested was prepared in a minimal salt solution to the approximate turbidity of a barium chloride standard for a Kirby-Bauer test (1) and swabbed onto the surface of the agar. Amino acid-saturated disks were placed on the agar surface with sterile forceps. After incubation for 48 h at 36°C in 8 to 10% CO_2, additional requirements could be identified by growth around a disk (Fig. 2). When such a requirement was identified, that amino acid was then added to the medium of the auxotyping series.

Miniplate procedure for auxotype and confirmation. A 15- to 18-h growth of the strain to be tested was suspended in 2.0 ml of minimal salts solution to the approximate turbidity of the barium chloride standard previously described. A loop calibrated to deliver 1 μl was used to inoculate each test well. The plates were incubated in an 8 to 10% atmosphere of CO_2 at 36°C for 48 h. After incubation, a drop of 0.1% solution of triphenyltetrazolium chloride was added

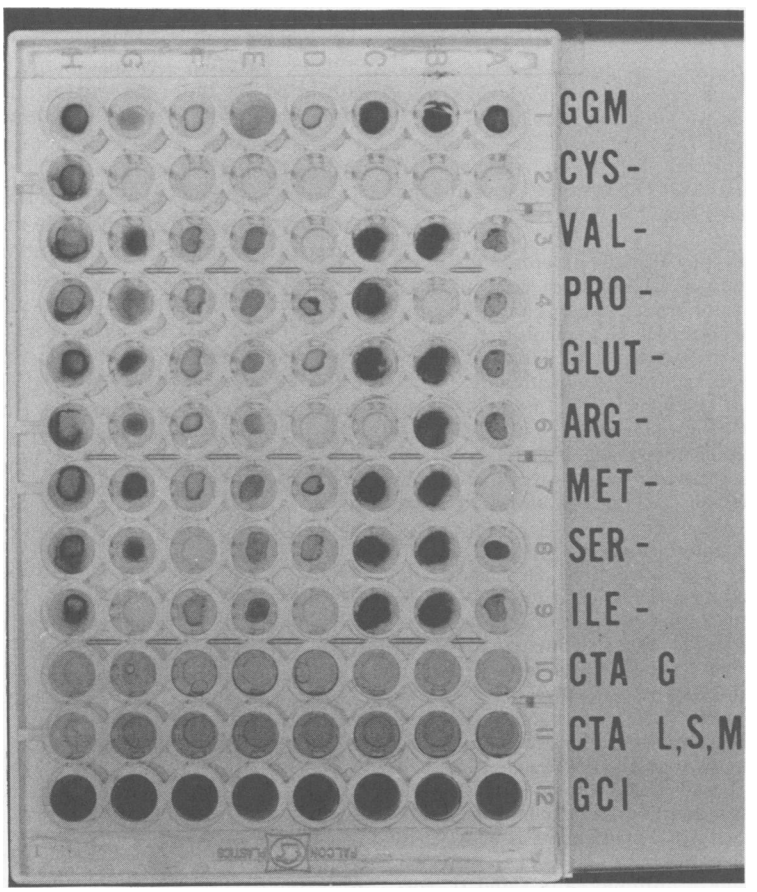

FIG. 1. *Analysis of auxotypes by miniplate procedure. Eight strains were inoculated on the miniplate as described in the text. Rows A through G are different isolates of N. gonorrhoeae; row H is a strain of Neisseria meningitidis. Rows 1 through 12 are as follows: (1) complete GGM; (2) complete GGM minus cysteine/cystine; (3) complete GGM minus valine; (4) complete GGM minus proline; (5) complete GGM minus glutamic acid; (6) complete GGM minus arginine; (7) complete GGM minus methionine; (8) complete GGM minus serine; (9) complete GGM minus isoleucine; (10) CTA-glucose; (11) CTA-lactose-sucrose-maltose; (12) GCI with oxidase reagent added. Note that all N. gonorrhoeae require cysteine. Auxotypes appear as follows: strain A, Met; strain B, Pro; strain C, Arg; strain D, Val, Arg, Ile; strain E, wild type; strain F, Ser; strain G, Ile; strain H, N. meningitidis.*

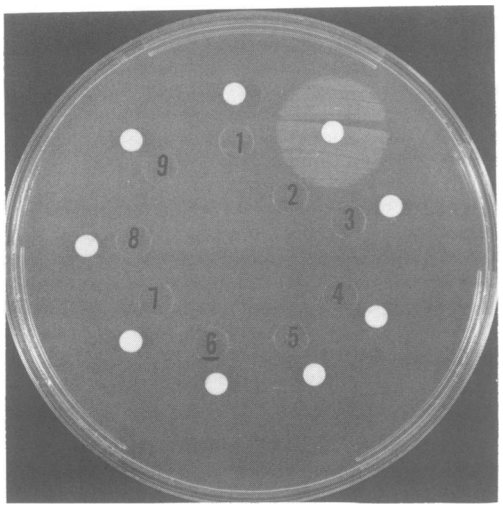

FIG. 2. *Identification of additional auxotypes. Complete GGM was supplemented with disks containing 200 μg of the following amino acids: 1, L-lysine; 2, L-tyrosine; 3, L-tryptophan; 4, L-leucine; 5, L-threonine; 6, L-alanine; 7, L-glycine; 8, L-phenylalanine; and 9, L-histidine. The strain illustrated has a requirement for L-tyrosine.*

to wells 1 through 9 by Pasteur pipette to facilitate reading. Utilization of CTA-glucose was indicated by a change in color from red to yellow. Because some acidification occurred due to incubation in CO_2, the CTA sugar utilization determinations were made 1 h after removal from the incubator.

Oxidase reagent (1% solution of dimethyl-*p*-phenylenediamine-HCl) was added to the GCI well. Confirming Gram stains were made after oxidase testing. Stains were tested at least two times by the miniplate method as well as the standard plate method.

DNA-mediated transformation. Type 1 (8) recipient strains were inoculated onto GCI agar and grown for 18 to 20 h at 37°C in 8 to 10% CO_2. Cells were harvested in a 2.5-ml suspension in equal parts of minimal salts I and II (10) and centrifuged at 3,000 × *g* for 7 min, and the pellet was resuspended in minimal salts I and II. Two procedures were used to measure the frequency of transformation. The frequency of transformation could be determined simply by adding recipient cells to GCI agar that had previously been spread with 0.2 ml of a crude DNA lysate. DNA was isolated by the procedure of Janik et al. (6). After 4 to 6 h of incubation at 37°C to permit phenotypic expression, the cells were transferred to a solution of minimal salts I and II, vortexed, and transferred to GGM minus proline or GCI containing 1 μg of rifampin per ml. The viable count was determined on GCI. Alternatively, transformation could be accomplished by incubating 0.8 ml of recipient cells plus 0.1 ml of 10 mM $MgCl_2$ and 0.1 ml of crude DNA lysate. Selection was accomplished by directly plating samples on GGM minus proline or GCI. After 4 h at 37°C, rifampin-resistant clones were selected by a 2.0-ml overlay of rifampin (1 μg/ml). The frequency of transformation was similar with both of these methods.

The concentration of DNA in the crude lysate was determined by the diphenylamine procedure of Burton (2).

RESULTS

Analysis of the growth of clones on miniplates demonstrated a number of different auxotrophic requirements. All but 2 of the isolates from 40 patients could be classified on GGM or GGM supplemented with additional amino acids as described above. The most common additional requirement was L-histidine; however, in a larger study L-leucine was the commonest additional growth requirement (13). The isolate requiring L-tyrosine is the only one of its type in our laboratory from any study. The response of gonococci to isoleucine and valine is variable. Because some strains can be inhibited by high concentrations of isoleucine or valine, it is important to maintain precise concentrations of these supplements in auxotyping media.

Auxotyping of 8 clones from the remaining 38 patients was performed by the miniplate procedure and compared with standard auxotyping techniques (10). Identical results were obtained with both methods. The relative frequency of auxotypes occurring as a single phenotype is shown in Table 1. Nine patients showed two different auxotypes in the eight clones selected, and one specimen contained three auxotypes (Table 2). Because gonococci are competent throughout their entire growth period (7) and could, therefore, transform each other, it is difficult to determine merely by auxotyping whether there is a mixed infection, or if reversion occurred at a locus with subsequent transformation of the population. Leucine, isoleucine, and valine markers revert to prototrophy with a relatively high reversion rate, whereas proline and arginine markers rarely revert. It was possible that the auxotrophic differences

TABLE 1. *Phenotypes of specimens with eight identical clones*

Phenotype	No.
Wild type[a]	12
Arg	5
Pro	4
Val Ile	2
Arg Val Ile	2
Arg Val Ile His	2
Leu	1

[a] Wild type is defined only in relationship to amino acid growth requirements. Since all *N. gonorrhoeae* require cysteine or cystine for growth, these strains are designated wild type.

TABLE 2. *Specimens with two or more phenotypes from eight clones*

Patient no.	Phenotype	No. of clones
1	Wild type	4
	Ile	4
2	Pro	7
	Arg Val Ile	1
3	Arg Met	1
	Arg Ile	4
	Tyr	3
4	Wild type	7
	Arg Ile	1
5	Arg Ile	2
	Arg Ser Ile	6
6	Pro	4
	Pro Ile	4
7	Wild type	6
	Pro Met	2
8	Wild type	7
	Pro	1
9	Pro	5
	Pro Arg	3
10	Pro	5
	Pro Met	3

observed in patients 1, 5, 6, and 10 could have been due to reversion, but the differences in cultures from patients 2, 3, and 4 appeared to represent infection with multiple auxotrophs. Because all of the clones isolated from the two patients that yielded an organism that would not grow on GGM were similar, the maximum percentage of patients with more than one phenotype was 25%.

A further analysis of relatedness of the clones from patients 6, 9, and 10 can be accomplished with complementation analyses by DNA-mediated transformation. Two loci have been recognized in our laboratory that encode proline biosynthesis. As shown in Table 3, the laboratory strain F62 carries one of these loci, which we designated *pro-1*, and the strain H4 carries another, which we have designated *pro-2*. When F62 and H4 were used as recipients, it was clear that both auxotypes from patients 6, 9, and 10 had the same *pro* marker. The isolates from patient 6 complemented strain F62, whereas the isolates from patients 9 and 10 complemented strain H4. Similar results were obtained when the clinical isolates were used as recipients and strains F62 and H4 as donors. It was interesting to note that transformation of strain F62 by DNA from strain H4 resulted in a 2,000-fold increase in proline transformants as compared with rifampin-resistant transformants.

DISCUSSION

The procedure described permits a rapid, inexpensive auxotyping of clinical isolates. Because the medium is stable at 4°C for at least 2 months, this method can readily be adapted to the clinical laboratory. Because of the relatively high incidence of multiple auxotypes, auxotyping is less valid when only one clone is tested from a single clinical specimen. Although it is conceivable that some of the differences result from reversion of more unstable loci, it is evident that some patients (such as patient 3 in Table 2) carry multiple strains. A similar occurrence of multiple infections was observed in patients with *Salmonella*. In Juenker's series, 13 of 75 patients carried multiple serotypes (7).

If required, further classification of isolates can be done by DNA-mediated transformation. For instance, there are at least eight classes of naturally occurring arginine-requiring mutants and two classes of proline-requiring strains (13). Because these variants are frequently present in clinical isolates, DNA-mediated transformation could provide an aditional method for establishing identity of loci within the organism from patients with alleged multiple strains. Analysis of the Pro locus of the seemingly different auxotypes in the clones isolated from patients 6, 9, and 10 was done, using two standard Pro recipients, *pro-1* and *pro-2*. Complementation experiments demonstrated that the Pro loci in each of the eight clones obtained from each patient was identical. DNA from strains obtained from patient 6 complemented the *pro-1* locus, whereas those of patients 9 and 10 complemented the *pro-2* locus.

Additional rapid procedures for classification could be introduced into the miniplates, such as

TABLE 3. *Analysis of Pro markers*

DNA donor	Recipients (transformants/ml)			
	F62		H4	
	Pro^+	Rif^r	Pro^+	Rif^r
Patient[a]				
2A	8.8×10^5	2.0×10^4	$<10^1$	2.6×10^4
2B	7.9×10^5	8.0×10^3	$<10^1$	1.1×10^4
6A	$<10^1$	1.2×10^4	2.1×10^3	5.5×10^4
6B	$<10^1$	1.9×10^4	2.7×10^3	2.2×10^4
9A	$<10^1$	1.2×10^5	2.8×10^4	8.9×10^4
9B	$<10^1$	2.2×10^4	8.0×10^2	9.5×10^3
10A	$<10^1$	2.3×10^3	6.4×10^3	1.0×10^4
10B	$<10^1$	2.0×10^4	1.0×10^3	4.2×10^3
Strain				
H4	3.9×10^5	1.8×10^2	$<10^1$	1.7×10^3
F62	$<10^1$	2.3×10^3	2.2×10^4	5.0×10^3

[a] Clones from a patient were numbered A through H.

the pyocins recently described by Morse and coworkers (11), antibiotic susceptibility (12), or the inhibitory factor(s) isolated from yeast (5). Due to the frequency of multiple infections, it is important to analyze more than one clone in careful epidemiological studies. Finally, it is important to stress Catlin's original observation that gonococci, unlike most meningococci, require cysteine/cystine for growth (4). This test has been helpful in our laboratory in distinguishing meningococci from gonococci. Studies are in progress to determine whether selective pressures exist that influence the prevalent auxotype in different communities.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service grant 1 R01-AI-11709 from the National Institute of Allergy and Infectious Diseases.

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