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Microbial Population Diversity in the Urethras of Healthy Males and Males Suffering from Nonchlamydial, Nongonococcal Urethritis

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Nonchlamydial, nongonococcal urethritis (NCNGU) is suggested to be a sexually transmitted disease in men. NCNGU patients were compared to control subjects with regard to the presence of potentially infectious bacteria in the first void urine. Patients' pre- and post-antibiotic-treatment urine samples and two samples obtained 2 weeks apart from healthy volunteers, who did not receive antibiotic therapy, were analyzed with broad-spectrum PCR tests aiming at eubacterial small subunit rRNA genes. Restriction fragment length polymorphism analysis of the amplicons cloned from the mixtures of PCR products revealed that many different species of microorganisms were found to be colonizing the male urethra. We document here clear differences in the composition of the resident urethral flora between samples obtained from various individuals and between samples obtained at various points in time for a single individual. No major changes in population complexity were found upon antimicrobial treatment. In two of five patients a previously suggested pathogen (*Mycoplasma genitalium* or *Haemophilus parainfluenzae*) was accurately identified on the basis of DNA sequencing. No ubiquitous, azithromycin-sensitive organism was identified as a common pathogen in all patients, but up to 40% of all clones represented as-yet-unclassified bacterial species. Relatively often *Pseudomonas* spp. or *Pseudomonas*-like organisms were identified in the bacterial flora of patients. Interestingly, an as-yet-uncharacterized microbial species was identified as a negative predictor of NCNGU. This species was identified in all control subjects and was absent from all of the patient' samples (5 of 5 versus 0 of 5, $P = 0.0079$). This suggests that NCNGU might also be diagnosed by assessing the absence rather than the presence of certain bacterial species.

Chlamydia trachomatis and *Neisseria gonorrhoeae* are common causes of urethritis in males (7, 14, 16). Nongonococcal urethritis is diagnosed in over two million cases per year in the United States. A significant fraction of the urethritis patients (up to 50%), however, are not infected by either one of these pathogens (7). In these cases, the clinical syndrome is referred to as nonchlamydial nongonococcal urethritis (NCNGU). NCNGU is a common condition, frequently diagnosed in sexually transmitted disease (STD) clinics around the world. In the pathogenesis of acute NCNGU, microorganisms other than *C. trachomatis* or *N. gonorrhoeae* seem to play a role as well, since a significant fraction of the patients involved respond well to antibiotic treatment (1). Furthermore, data showing that condoms are protective against NCNGU support the hypothesis that NCNGU is an infectious disease (8, 25). Despite extensive microbiological studies, no single causative microbial species has been identified as the main cause of NCNGU. Although *Mycoplasma genitalium* seems to be an important candidate (4, 14, 17, 20, 28), with an incidence that may be as high as 36% (4), many clinically overt cases still remain microbiologically unexplained. Previous studies were limited to selective searches for one or more traditional pathogens that are putatively involved in inflammation of the lower genital tract in males. These pathogens included *M. genitalium*, *Ureaplasma urealyticum*, or *Trichomonas vaginalis*, and searches were performed by culture, PCR, or a combination of both methods (14, 20, 28). Efforts to really identify novel pathogens have employed culture methods mainly (10, 26). The downside of this approach is that culture-dependent techniques may not adequately elucidate the microbial diversity in the genitourinary tract. Molecular techniques have suggested that the number of pathogenic microorganisms that have been cultured to date probably equals only a fraction of the total (23). Consequently, culture-based approaches have probably prohibited the discovery of novel uropathogens, an omission that underscores the need for additional molecular-microbiological searches.

A potential solution to the inadequacy of microbiological culture appears to be the use of diagnostic broad-range ribosomal DNA (rDNA) amplification in combination with phylogenetic studies (21). Eubacterial, domain-specific PCR primers are particularly useful for the identification of putative human pathogens. Amplified rDNA from bacteria can be sequenced, and these sequences can be used in computerized database searches to identify the bacterial species involved (31). This approach turned out to be particularly successful in microbial-etiological studies in chronic idiopathic prostatitis. Bacterial rRNA genes could be detected in 77% of all patients, and certain *Vibrio* species were identified as putative agents of infection (19). In addition, the same strategy helped to identify novel bacterial species, both in natural environments and clinical syndromes (3, 11, 15, 22, 29).

The objective of the current research was to define the

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TABLE 1. Age, STD history, and clinical findings of patients and controls

Patient or control no.	Age (yr)	STD history[a]	First visit		Second visit		Sexual orientation
			Symptoms	PMNs/μl	Symptoms	PMNs/μl	
Patient							
1	37	NG	Discharge, dysuria	21	None	5	Heterosexual
2	29	CT, PB	Discharge, dysuria	31	None	5	Homosexual
3	25	CT, GW	Dysuria	107	None	5	Heterosexual
4	32	NG, CT	Dysuria	21	None	2	Heterosexual
5	44	NG, NCNGU	Dysuria	61	None	1	Homosexual
Control							
1	36	NCNGU	None	1	None	1	Homosexual
2	30	PB	None	0	None	3	Homosexual
3	50	None	None	2	None	1	Heterosexual
4	29	None	None	2	None	2	Heterosexual
5	40	None	None	4	None	1	Heterosexual

[a] NG, *N. gonorrhoeae*; CT, *C. trachomatis*; PB, pubic pediculosis; GW, genital warts. All patients suffered from penile irritation.

microbial communities present in the urethra of healthy male volunteers and NCNGU patients. We searched for putative pathogens and/or markers for a healthy microbial flora by ribosomal PCR, which should allow for a detailed comparison of the flora of control individuals and the spectrum of bacterial species present in pre- and post-antibiotic-treatment samples of NCNGU patients.

MATERIALS AND METHODS

Participants and procedures. Men attending the STD Outpatient Clinic of the Department of Dermatology and Venereology of the Erasmus MC (University Medical Centre Rotterdam, Rotterdam, The Netherlands) for a sexual health assessment were eligible for the present study. Selected individuals provided informed consent (Erasmus MC Medical Ethical Committee, protocol 00-859). Major exclusion criteria were the use of antibiotics within the previous month and a history of urethritis within the previous 3 months. Personal interviews revealed that all five enrolled patients had clear symptoms of urethritis (penile irritation in combination with discharge and/or dysuria [see Table 1]). Urethritis was confirmed microscopically, on the basis of >6 polymorphonuclear leukocytes (PMNs) per μl in the sediment of 12 ml of first-pass urine (FPU). The numbers of PMNs were determined by using the standardized KOVA system (Hycor, Garden Grove, Calif.) in full accordance with the manufacturer's instructions. Control subjects were five asymptomatic volunteers with no signs of urethritis (≤6 PMNs/μl). After the urethral meatus of each patient was washed with a sterile gauze and tap water, ~30 ml of FPU was collected into sterile tubes. In addition, urethral swabs were obtained for confirmatory purposes. Infection by *N. gonorrhoeae* was excluded microscopically (classical Gram stain) and by culture performed on a GC-LECT agar (Becton Dickinson, Alphen aan den Rijn, The Netherlands). *C. trachomatis* infection was excluded by PCR analysis of FPU by using the Cobas Amplicor Detection Reagent Kit and the Cobas Amplicor machine (Roche Diagnostics, Mannheim, Germany) according to instructions of the manufacturer. A total of 12 ml of FPU was used for the microscopic and diagnostic evaluations described above, whereas 500 μl was used for the Amplicor tests. In preparation of the broad-spectrum ribosomal PCRs, 10 ml of FPU was centrifuged for 10 min at 3,000 rpm. The sediment (ca. 75 to 300 μl) was kept at −80°C prior to processing. Patients with microscopically diagnosed urethritis and a negative Gram stain for *N. gonorrhoeae* were treated with a single oral dose of 1 g of azithromycin. Patients and control subjects were advised to abstain from any form of sexual intercourse (vaginal, anal, and oral) and were asked to return for reexamination after 2 weeks. At the second visit they delivered ca. 30 ml of FPU, which was collected at home in the early morning, according to the collection procedure described above. Again, 12 ml of FPU was microbiologically evaluated for the presence of PMNs. A 10-ml portion was centrifuged, and the sediment was kept at −80°C prior to PCR processing. The healthy volunteers were not treated with azithromycin between the two samplings.

Tap water. To determine the possible microbiological contamination factor of tap water used for washing the urethral meatus, 50 ml of first-run tap water was collected in a sterile tube. This was centrifuged in 10-ml parts for 10 min at 3,000 rpm. The sediment was kept at −80°C prior to processing.

DNA purification. Part of the collected water and urine sediments (150 μl) was used for DNA extraction and purification. To the samples with a volume of <150 μl, a compensating amount of 50 mM Tris-HCl (pH 7.5)–0.1 mM EDTA–50 mM glucose buffer was added to a total volume of 150 μl. First, 75 μl of lysostaphin solution (10 mg/ml; Sigma, St Louis, Mo.) was added, and the mixture was heated to 37°C for 30 min. Thereafter, 1 ml of guanidinium lysis buffer (4 mM guanidinium isothiocyanate, 0.1 M Tris-HCl [pH 6.4], 0.2 M EDTA, 0.1% Triton X-100) was added, and the mixture was kept at room temperature for 1 h, after which 50 μl of Celite suspension was added. The samples were kept at room temperature and mixed at regular intervals for 10 min (5). After a vortexing step and centrifugation (15 s at 14,000 rpm in an Eppendorf centrifuge), the supernatant was discarded and the pellet was washed twice with a second chaotropic lysis buffer (4 M guanidinium isothiocyanate, 0.1 M Tris-HCl; pH 6.4), twice with ethanol (70%) and, finally, once with acetone. The pellet was vacuum dried and emulsified in 100 μl of 10 mM Tris-HCl (pH 8.0). The sample was heated to 56°C for 10 min and centrifuged (10 min at 14,000 rpm in an Eppendorf centrifuge). The resulting supernatant was used as a template for PCR.

PCR tests. All PCRs were performed in GeneAmp 9600 or 9700 machines (PE Applied Biosystems, Foster City, Calif.). The primers used for the 16S rDNA PCR were EUB-L (5′-CTTTACGCCCATTTAATCCG-3′) and EUB-R (5′-AGA-GTTTGATCCTGGTTCAG-3′). These generate an ~500-bp fragment deriving from the 3′-terminal end of the small-subunit (ssu) rRNA gene (30). A total of 45 μl of PCR mix was added to 5 μl of the purified DNA solution. The PCR mix consisted of 10 μl of a 20 mM desoxynucleotide triphosphate stock solution (Amersham Life Science, Cleveland, Ohio), 5 μl of a 10-fold-concentrated SuperTaq PCR buffer (HT Biotechnology, Cambridge, United Kingdom), 0.5 μl of both primers, 28.92 μl of distilled water, and 0.08 μl of SuperTaq polymerase (15 U/μl; HT Biotechnology, Cambridge, United Kingdom). The PCR consisted of 40 cycles of denaturation at 94°C (45 s), annealing at 55°C (45 s), and extension at 72°C (45 s). A precycling denaturation step at 94°C was applied for 5 min. As control sample, 50 μl of PCR mix without additional DNA samples was run in parallel. Then, 10-μl portions of the PCR products were analyzed on a 1% agarose gel containing ethidium bromide. Electrophoresis was performed in 0.5× TBE (50 mM Tris, 50 mM borate, 1 mM EDTA); gels were then stained in aqueous ethidium bromide (10 ng/ml) and photographed under UV illumination.

Cloning of amplification products. The PCR amplification products (3 μl of a PCR mix) were used for ligation in pCR2.1 and transformed into competent *Escherichia coli* TOP10 cells by using the Original TA Cloning Kit (Invitrogen, San Diego Calif.). Clones were grown overnight at 37°C on 2YT agars (Yeast-Trypton; Gibco-BRL, Breda, The Netherlands) containing ampicillin (100 μg/ml) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 40 μg/ml). Possible transformants were identified by blue-white colony screening.

Screening for full-length inserts. DNA was liberated from possible recombinants by suspending part of the colony in 100 μl of distilled water. The suspension was boiled for 10 min and centrifuged for 10 min at 14,000 rpm in an Eppendorf centrifuge. Next, 5 μl of the supernatant was used as a template for PCR. The primers used for amplification of the putative inserts in pCR2.1 were M13 and T7 sequence specific (AACAGCTATGACCATG and TAATACGAC TCACTATAGGG, respectively). then, 45 μl of PCR mix, identical to the mix used for the ssu rDNA PCR, was added. The PCR consisted of 30 cycles of denaturation at 94°C (45 s), annealing at 56°C (45 s), and extension at 72°C (45

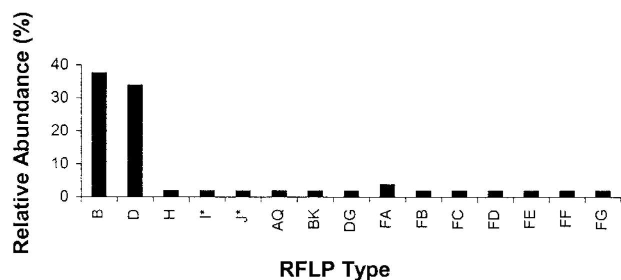

FIG. 1. Distribution of ssu rDNA PCR RFLP types derived from tap water. Indicated are the *Alu*I types identified by uppercase letters versus their frequency of occurrence among the 55 clones studied. Note that types B and D, representing *Pseudomonas* spp., are by far the most prevalent. The relative abundance represents the proportion the number of representatives of a given RFLP type divided by the cumulative number of all clones analyzed. The latter is set at 100%.

s). A precycling denaturation at 94°C was applied for 5 min. The PCR products were visualized as described above. Only the samples with a full-length insert (~700 bp) were analyzed by restriction enzyme digestion. Per clinical sample, ca. 50 clones with full-length inserts were selected for further analysis.

RFLP analysis. We digested 15 μl of the PCR product solution (M13/T7 PCR) by using the restriction endonuclease *Alu*I (New England Biolabs, Beverly, Mass.). The restriction digests were analyzed in a 3% Metaphor agarose gel containing ethidium bromide. The electrophoresis was performed in 0.5× TBE. The gels were stained, examined, and photographed under UV illumination. Analysis of the different restriction fragment length polymorphism (RFLP) patterns was initially performed visually. If it was not possible to discriminate between certain types, the software program GelCompar version 4.0 was also used. When GelCompar was used, the position of DNA fragments shorter than 100 bp was ignored because these were not resolved well enough. For the remaining DNA fragments, bands were analyzed according to Dice with the tolerance set at 1.0% (optimization = 0.50%, minimal area = 0.1%). Two RFLP patterns were regarded as the same if they matched for 100%. Different RFLP types were given separate capital letter codes.

DNA sequencing. For bidirectional sequencing of the insert, the ssu rDNA PCR (described above) was repeated. The nucleic acid sequence of the PCR product was analyzed by Sanger's method (BaseClear, Leiden, The Netherlands) by using the Big Dye terminator sequencing kits 373, 377, and 3100 (PE Applied Biosystems). The assembled ssu rDNA sequences were subjected to basic local alignment search tool (BLAST) analysis (http://www.-ncbi.nlm.nih.gov/blast/; version 1 June 2002). This analysis was used to determine which sequence in the GenBank depository was most similar to the partial 3′-terminal ssu rDNA sequence of the isolate. For the construction of phylogenetic trees, the sequence data were compared by using multiple sequence alignment software as available at www.genebee.msu (A. N. Belozersky Institute, Russian EMB Net Node) and were expressed as phylograms (6).

RESULTS

Participants. The five patients had a mean age of 33 years; the mean age of the controls was 37 years (Table 1). Although patients were asked to return 2 weeks after the initial visit, logistical difficulties caused the interval time to vary between 13 and 22 days. The control subjects all presented with an interval time of precisely 14 days. All patients had a history of some sort of urethritis (chlamydial, gonococcal, or NCNGU) in the years prior to the study. In comparison, only one of the control subjects suffered from NCNGU in the past. Patients and control subjects refrained from sexual intercourse between visits, had negative tests for *N. gonorrhoeae* and *C. trachomatis*, and did not have signs of herpes genitalis or other overt sexually transmissible diseases. Table 1 presents the urine PMN cell counts, thereby illustrating that for all of the patients the NCNGU episode cleared between the two visits, probably due to the azithromycin therapy. Clinical complaints resolved during the 2-week period as well. The cell counts for the controls were always in the normal range (≤6).

RFLP analysis of ribosomal clones derived from tap water sediments. Many bacterial ribosomal clones were obtained by cloning the PCR products of the sediment of 50 ml of tap water. Upon analysis of 55 clones, 15 clearly different RFLP types were documented (see Table 2). Figure 1 shows the distribution of these clones across the RFLP types, indicating their relative frequencies of occurrence. There were seven RFLP types (FA to FG) present that were never obtained from controls and patients. Types B and D appeared to be most common. Sequencing of some of the clones revealed that these two types represented *Pseudomonas* spp., which are known to be associated with water-rich environments. The amounts of bacteria present in tap water probably mask the detection of rDNA contaminants in the reagents used for PCR.

RFLP analysis of ribosomal clones derived from urine sediments. Table 2 indicates the number of clones that were analyzed for patients and volunteers by the RFLP approach. In all, RFLP patterns were recorded for 472 clones derived from patient samples, and 488 were documented for the healthy controls. Ultimately, the RFLP database is comprised of 960 ribosomal fingerprints. *Alu*I digestion of full-length inserts generally resulted in two to five conveniently resolved bands (Fig. 2). Different RFLP types represent the different types of ssu rRNA genes that are present in microbes concentrated in the urine sediment. It has to be emphasized, since we only employed a single restriction enzyme, that clones with identical RFLP patterns do not necessarily represent identical bacterial species. The distribution of clones (both in RFLP type and

TABLE 2. Clones and RFLP types recovered from water, control, and patient samples

Control no. sample type, or patient no.	Visit	No. of clones analyzed	No. of RFLP types
Controls			
1	First	55	22
	Second	50	15
2	First	46	19
	Second	62	22
3	First	49	15
	Second	50	22
4	First	49	14
	Second	43	18
5	First	48	15
	Second	36	11
Water[a]	Single[a]	55	15
Patients			
1	First	44	10
	Second	49	15
2	First	50	11
	Second	49	14
3	First	46	28
	Second	37	15
4	First	48	8
	Second	49	11
5	First	50	18
	Second	50	21

[a] The water sample was obtained only once.

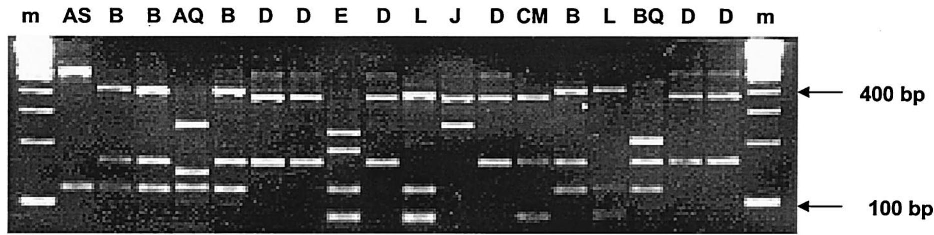

FIG. 2. PCR RFLP analysis of a randomly selected subset of the ssu rDNA clones obtained from the urine sediments of patient 1. Both on the right and on the left, a molecular size marker is included (100-bp ladder; Bio-Rad, Veenendaal, The Netherlands); above the lanes, the uppercase letter code for the RFLP patterns is indicated.

abundance) in control subjects and in patients is plotted in Fig. 3 and 4. Overall, 71 and 84 different RFLP types were obtained in control subjects and patients, respectively. We found 62 different RFLP types present in patient samples that were absent in control samples (CA-EJ). All clone libraries had a relative dominance of a few types (e.g., the "watertypes" B and D in most samples or CV and DE in the sample obtained during the first visit of patient 2). It can be noted that some types were only present during one visit (e.g., in control 4, types Q and BF). Also, most of the types were not found in all of the

Control 1

Control 2

Control 3

Control 4

Control 5

Relative Abundance (%)

RFLP Type

FIG. 3. Distribution of ssu rDNA PCR RFLP types derived from control individuals. Open bars indicate the clones identified from the urine sediment obtained during the first visit; solid bars indicate clones from the sediment collected at the second visit. On the horizontal axis the RFLP types are identified; on the vertical axis the relative abundance of the types is shown. For a definition of relative abundance, see the legend to Fig. 1. Codes indicated by an asterisk were hard to classify definitely and may represent heterogeneous types probably consisting of more than one sequence motif.

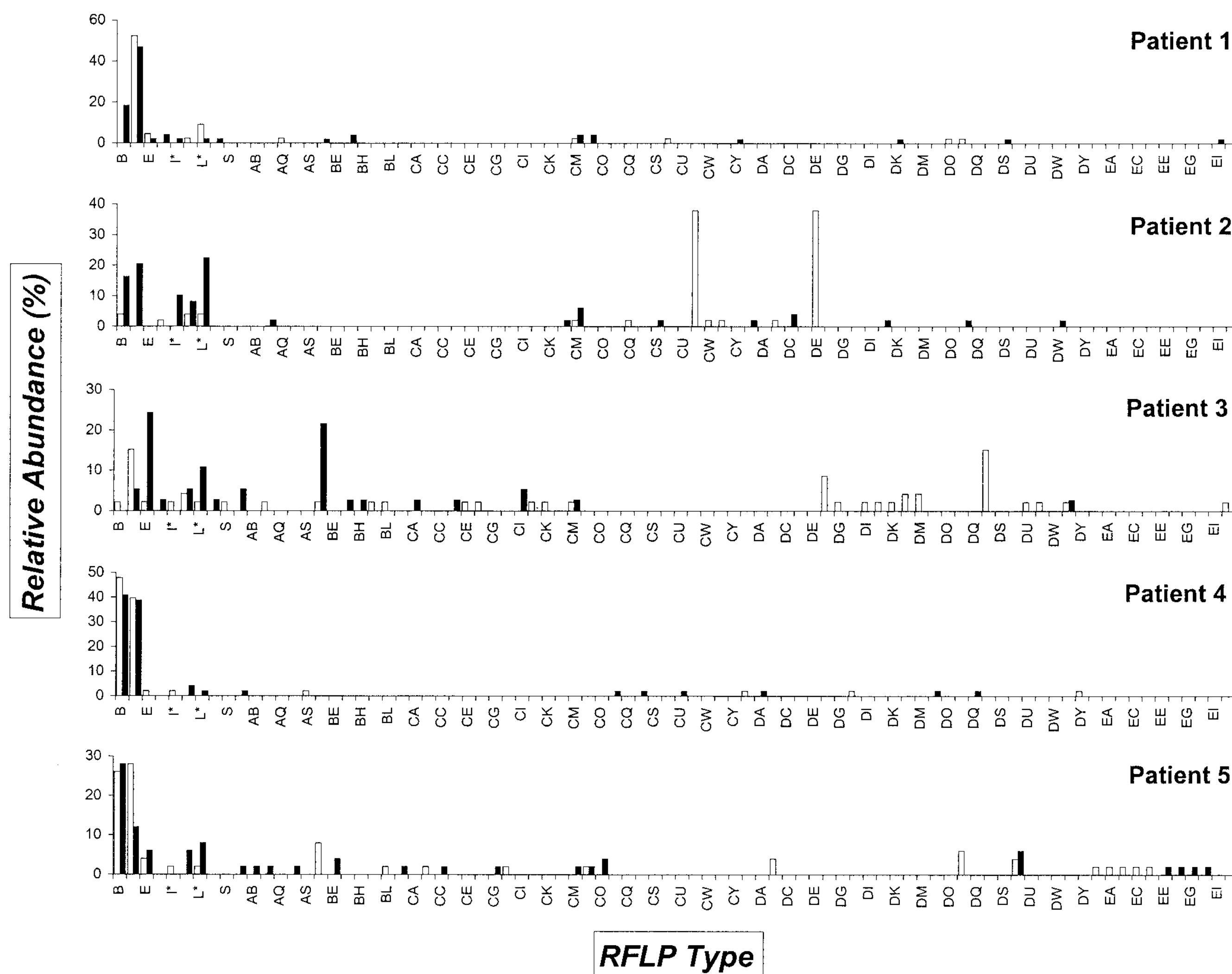

FIG. 4. Distribution of ssu rDNA PCR RFLP types derived from the five patients. Open bars indicate the clones identified from the urine sediment obtained during the first visit; solid bars indicate clones from the sediment collected at the second visit. On the horizontal axis, the RFLP types are identified; on the vertical axis, the relative abundance of the types is shown. For a definition of relative abundance, see the legend to Fig. 1. Codes indicated by an asterisk were hard to classify definitely and may represent heterogeneous types probably consisting of more than one sequence motif.

persons (e.g., type E was only present in control 1 and 3 and all patients except number 2). Especially noteworthy is the fact that RFLP type H was encountered in 9 of 10 samples derived from control individuals. During NCNGU, this type was never detected. This difference was highly significant from the statistical viewpoint (9 of 10 versus 0 of 5; $P = 0.0020$). Even when calculated for the two groups of individuals, this significance was maintained (5 of 5 versus 0 of 5; $P = 0.0079$). For RFLP type AQ, a similar trend was observed; in this case statistical relevance was not reached.

On average, the fraction of variant clones identified at any sampling moment was comparable. The index of variation, i.e., the ratio between the number of RFLP types and the overall number of clones studied, was between 0.32 and 0.36. These results indicate that the urethral flora shows extensive intra- and interpersonal variation. It is interesting that in the volunteers, 47 of 138 cumulatively identified types disappeared during the 2-week monitoring period. In the patients, who were treated with antibiotics, 50 of 127 identified types seemed to disappear. The difference in microbial dynamics between the two groups is not statistically significant (two-sided Fisher exact test, $P = 0.37$). This implies that the azithromycin treatment does not seem to drastically induce microbial species extinction in the urethra: in both controls and patients, similar elimination rates are encountered. On the other hand, species also appear during the monitoring period. Again, no statistically relevant difference is noted between the groups (52 of 138 versus 51 of 127; $P = 0.71$). In conclusion, azithromycin treatment does not seem to severely affect the composition and dynamics of the urethral flora. Interestingly, however, there still is a relatively large group of ssu rRNA PCR RFLP types that only occur in patients and not in controls ($n = 62$). A number of these disappear during the 2-week posttreatment period ($n = 34$). We assume that these represent antibiotic-susceptible pathogenic bacteria and, therefore, these clones will be addressed specifically below (see Fig. 5).

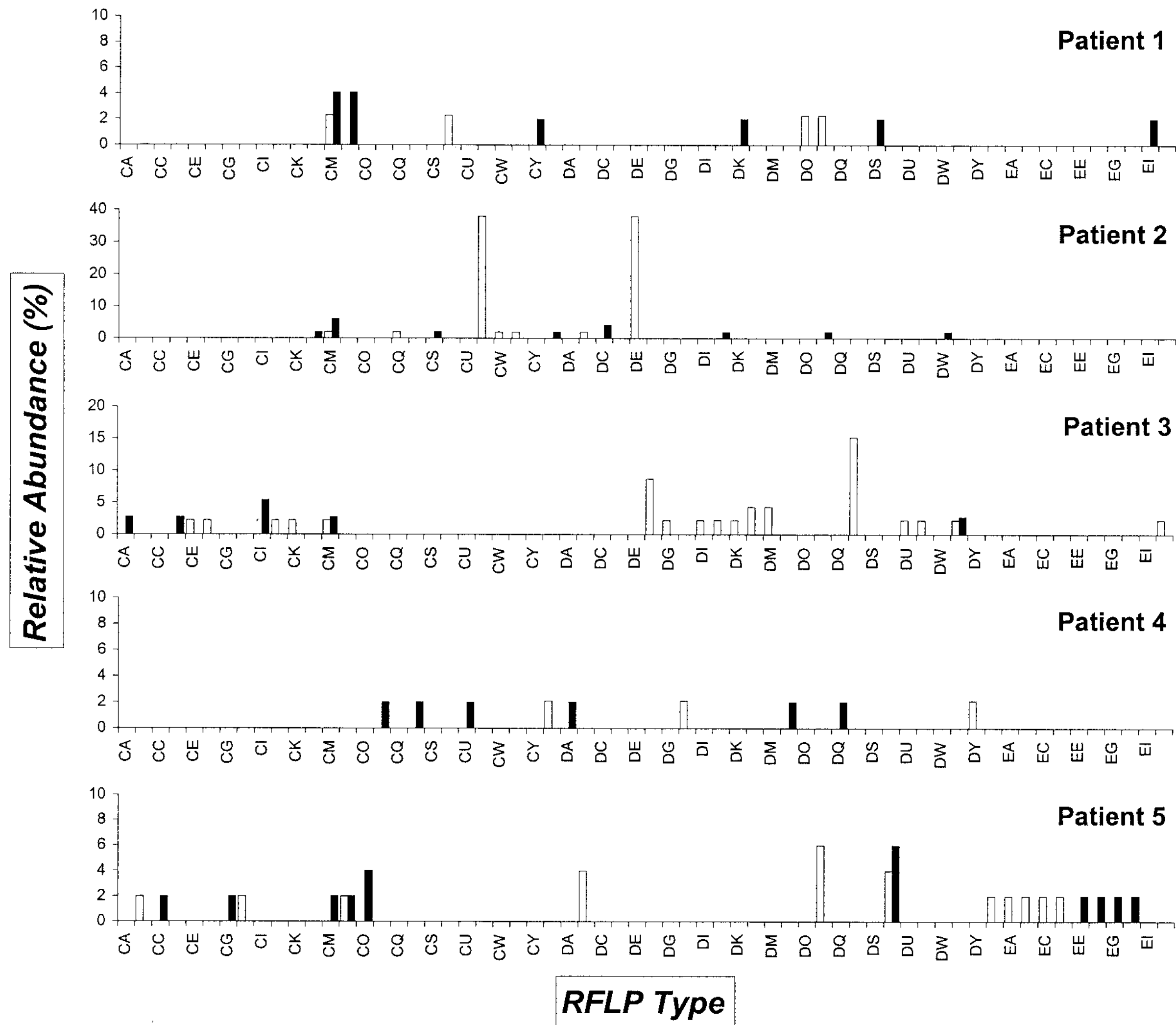

FIG. 5. Overview of the 16S rDNA RFLP types that are present in the patients and absent from the controls. Open bars indicate the clones identified from the urine sediment obtained during the first visit ($t = 0$); solid bars indicate clones from the sediment collected at the second visit ($t = 1$). On the horizontal axis, the RFLP types are identified; on the vertical axis, the relative abundance of the types is shown. For a definition of relative abundance, see legend to Fig. 1. Table 4 shows the sequence-based identification of a selection of these clones.

DNA sequencing. Due to the large number of different RFLP types (140 in all), sequence analysis of all 16S rDNA types was impractical. Some of the RFLP types found more frequently (relative abundance of >10%) were sequenced to assess the nature of the constant factors in the bacterial urethral flora in more detail. Table 3 surveys the resemblance of some of the more dominant clones with known bacterial species based on BLAST searches in GenBank. All sequencing analyses involved two independent clones per RFLP type. The fact that in eight of nine cases identical or highly similar sequences were obtained corroborates the reliability of the RFLP analysis. It should be noted that clones with different RFLP types (e.g., B and D, CV and DE, etc.) sometimes revealed a close relatedness to the same bacterial genus. In conclusion, Table 3 highlights that many water-borne bacterial species, including *Pseudomonas*, *Ralstonia*, and *Sphingomonas* spp., were identified as possible members of the healthy male urethral flora, some of which are also identified as water contaminants. The latter observation raises the question of whether these species are tap water contaminants or genuine members of the male urethral flora.

To determine which ssu rDNA types could possibly be associated with NCNGU, clones that appeared to be differentially distributed among patients and controls were subjected to sequencing. Type H, which, significantly, more often occurs in the flora of healthy men, gave rise to ribosomal sequence motifs that were most homologous to that of an uncultured bacterial species encoded GKS2-124. GKS2-124 belongs to the group of the α-proteobacteria and was initially isolated from a German freshwater lake (12). No obvious homology with known pathogenic bacterial species was observed, the sequence showed a somewhat more distant homology to water-thriving organisms such as *Sphingomonas* spp. A similar, though not statistically significant, trend was also observed for type AQ, occurring in four of five healthy controls and just one patient.

TABLE 3. Sequencing results of RFLP clones dominating one or more of the samples obtained from healthy control subjects

RFLP type	Sequencing result	Score
B	*Pseudomonas gessardii*	1092
	Pseudomonas libaniensis	1092
	Pseudomonas synxantha	1092
B	*Pseudomonas libaniensis*	1067
	Pseudomonas gessardii	1067
	Pseudomonas synxantha	1065
D	*Pseudomonas fluorescens* ATCC 49642	852
	Pseudomonas fluorescens ATCC 17574	852
	Uncultured manure pit bacterium P320	852
	Unidentified γ-proteobacterium OM93	852
	Pseudomonas sp. clone NBO.1H	852
D	*Pseudomonas veronii*	1074
	Unidentified γ-proteobacterium	1061
E	*Streptococcus* sp. oral strain H6	1043
	Streptococcus sp. oral strain B5SC	1041
	Streptococcus sp. oral clone BW009	1037
E	*Streptococcus mitis*	1065
H	Uncultured bacterium GKS2-124	979
	Unidentified gamma proteobacterium	965
	Sphingomonas echinoides	955
H	Uncultured bacterium GKS2-124	981
	Unidentified γ-proteobacterium	973
	Sphingomonas echinoides	963
L	*Ralstonia pickettii*	850
	Ralstonia sp. strain APF11	850
	Burkholderia pickettii ATCC 27511	835
L	*Ralstonia pickettii*	850
	Ralstonia sp. strain APF11	850
	Burkholderia pickettii ATCC 27511	835
U	*Pseudomonas fluorescens* bv.	771
	Pseudomonas gessardii	771
	Pseudomonas libaniensis	771
	Uncultured bacterium GR-296.II.35	771
	Pseudomonas sp. strain IC038	771
	Unidentified bacteria	771
U	*Ralstonia* sp. strain APF11	1086
	Uncultured bacterium OSs75	1053
	Pseudomonas pickettii	1045
AQ	*Sphingomonas echinoides*	765
	Sphingomonas echinoides	759
	Uncultured bacterium GKS2-124	757
	Unidentified α-proteobacterium	757
AQ	Unidentified α-proteobacterium	997
	Uncultured bacterium GKS2-124	989
	Star-like microcolonies	977
	Sphingomonas echinoides	971
BC	*Streptococcus mitis*	1019
BC	*Streptococcus mitis*	1080
BF	*Streptococcus agalactiae*	807
	Streptococcus agalactiae	805
BF	*Streptococcus agalactiae*	1082
	Streptococcus agalactiae	1074
	Streptococcus agalactiae	1005

Sequence analysis was also performed for one or two clones with RFLP types that were present in the first samples provided by the patients but absent from the water sample, the patient's follow up samples, and both samples from the controls (Fig. 5). Of the 32 different types of clones, 24 were successfully sequenced (Table 4). Interestingly, patient 2 represents the only individual for whom a well-known putative uropathogen was identified. Four of the different RFLP clones were very similar to the rRNA sequence of *M. genitalium*, thereby providing evidence for the fact that this person was cured from an *M. genitalium* infection. This fact again illustrates that the experimental approach employed was solid and that the outcome is relevant. However, the diversity observed among the RFLP patterns is somewhat enigmatic since *M. genitalium* only harbors a single copy of the 16S rRNA gene. Whether this variation is due to mixed infections or PCR and cloning induced errors is subject of current investigation. Patient 3 appeared to be infected by *Haemophilus parainfluenzae* (clone DF/DL), a bacterial species that has been mentioned before, but less convincingly as *M. genitalium*, in relation to NCNGU (27). For the other patients, various previously identified bacterial species, but also some species that currently lack a detailed description, matched entries in the GenBank database.

To strengthen the pathogen identification efforts, 18 minor clones from the controls were also sequenced (Table 4). Interestingly, 10 of 24 clones from the patients and 7 of 18 clones derived from the healthy controls were matched to microbial species that have never been cultured in vitro or have not yet been precisely classified as a species. Since the homology scores of the clone sequences with, for instance, the uncultured bacterial clone DJAT-434, occurring in four of five patients versus one of five control subjects, are variable, it is quite possible that we detected here novel bacterial species (Table 4). Figure 6 illustrates the gross interrelatedness of the individual bacterial species that were identified in Tables 3 and 4. Besides two heterogeneous groups of various bacterial species (*Actinomyces*, *Veillonella*, *Corynebacterium*, and *Ralstonia* spp., etc.), three major clusters can be discerned. Cluster A represents the streptococci, whereas cluster B derives from multiple clones analyzed for the patient suffering from an *M. genitalium* infection. Cluster C is the most interesting one since it is built from four subclusters, one of which (C3) gathers the various *Haemophilus* spp. The larger clusters C1 and C2 primarily contain *Pseudomonas* spp. Interestingly, the DJAT-434 homologues are clustered in the C2 group (except for the DJAT-434 homologue sequence derived from RFLP type DH from patient 4), a group consisting mainly of *Pseudomonas*-like organisms. Apparently, the DJAT-434 homologues closely resemble *Pseudomonas* spp. This leads to the conclusion that as-yet-unidentified *Pseudomonas* species may play a significant role, either as inducers of NCNGU or as opportunists occupying the environmental niche created during the disease process.

DISCUSSION

With regard to the etiology of NCNGU in males, it is appropriate to distinguish between acute and "chronic" disease. Although no precise definition is available, NCNGU etiology is probably multifactorial (2, 14). In order to gain more insight in the pathogenesis of NCNGU in males, novel diagnostic approaches are mandatory (7). We focused here on acute cases of urethritis, i.e., those not caused by *N. gonorrhoeae* or *C. trachomatis*. To our knowledge, ours is the first study to approach the long-standing enigma of the etiology of acute NCNGU in men, employing broad-spectrum molecular-biological techniques, such as ssu rDNA PCR and subsequent RFLP and sequencing techniques. A downside of this approach is that eukaryotic pathogens, such as *T. vaginalis*, are not detected. These organisms are certainly important in a subset of NCNGU cases (7, 18). Second, the data could be influenced by the fact that some microorganisms are present in larger numbers or contain more ribosomal operons per genome than others, thereby facilitating their detection. Consequently, the

TABLE 4. Sequencing results of RFLP clones only present in patients' $t = 0$ samples or randomly selected from $t = 0$ samples of controls

Patient or control no.	RFLP	Sequencing result	Score
Patients			
1	CT	Uncultured *Veillonella* spp.	1096
	DO	Uncultured bacterium clone DJAT-434	797
		Uncultured γ-proteobacterium	797
2	CQ	*Streptococcus gordonii*	1068
	CV	*Mycoplasma genitalium*	1019
	CW	*Mycoplasma genitalium*	1080
	CX	*Mycoplasma genitalium*	1096
	DB	Uncultured bacterium clone DJAT-434	797
		Uncultured γ-proteobacterium	797
	DE	*Mycoplasma genitalium*	1088
3	CJ	Unidentified bacterium 6C	624
		Methylobacterium spp.	595
	CK	*Corynebacterium thomssenii*	1007
	DF	*Haemophilus parainfluenzae*	969
		Haemophilus paraphrophilus	946
	DI	Unidentified oral bacterium AP60-15	676
		Gemella haemolysans	670
	DL	*Haemophilus parainfluenzae*	763
		Pseudomonas spp.	728
	DM	*Actinomyces turicensis*	648
		Bacterial spp.	634
	DR	*Haemophilus paraphrophilus*	959
		Haemophilus parainfluenzae	959
	DU	*Corynebacterium genitalium*	946
		Uncultured *Corynebacterium* sp.	473
	DV	*Pseudomonas fluorescens*	1051
4	DH	Uncultured bacterium clone DJAT-434	735
		Uncultured γ-proteobacterium	735
	DY	*Pseudomonas fluorescens*	1082
5	CB	Uncultured bacterium clone TFBME10	613
		Pseudomonas sp. G2	613
	CH	*Variovorax* sp. strain HAB-30	920
	DB	Uncultured bacterium clone DJAT-434	706
		Pseudomonas sp.	706
	DP	Uncultured bacterium clone DJAT-434	706
		Uncultured γ-proteobacterium	706
	EB	Uncultured bacterium D29A	1063
		Streptococcus salivarius	1061

Patient or control no.	RFLP	Sequencing result	Score
Controls			
1	K	*Mesorhizobium loti*	932
		Rhizobium sp. strain CJ5	924
		Rhizobium loti	924
	M	*Pseudomonas marginalis*	1072
		Pseudomonas spp.	1072
		Pseudomonas reactans	1072
		Pseudomonas veronii	1072
	H	Unidentified bacterium ox-SCC-25/5	946
		Unidentified bacterium ox-SCC-36/29	930
	O	Uncultured *Comamonas* spp.	876
		Pseudomonas testosteroni	876
		Comamonas testosteroni	876
	P	*Peptostreptococcus* genospecies	500
		Peptostreptococcus spp.	456
	T	Uncultured *Comamonas* spp.	891
		Pseudomonas testosteroni	891
		Comamonas testosteroni	891
	AJ	Uncultured eubacterium WD293	1017
		Pseudomonas mephitica	1005
	AK	Uncultured eubacterium WD293	1036
		Agricultural soil bacterium	1027
	AN	*Pseudomonas* spp.	1052
		Pseudomonas gessardii	1046
		Pseudomonas libaniensis	1046
	G	Uncultured bacterium clone DJAT-434	783
		Uncultured γ-proteobacterium	783
2	BG	*Sphingomonas* spp.	787
		Uncultured bacterium GKS2-124	785
	AO	*Ralstonia detusculanense*	628
		Ralstonia spp.	628
		Uncultured bacterium OSs7	628
3	V	Unidentified bacterium rM7	648
		Comamonas testosteroni	640
	AE	*Ralstonia detusculanense*	713
		Ralstonia spp.	713
		Burkholderia spp.	682
	AF	*Pseudomonas jessenii*	653
		Pseudomonas spp.	653
		Uncultured eubacterium	647
	AG	*Pseudomonas* sp. strain G2	1036
		Pseudomonas sp. strain NZ66/64/124/122/113/108/106/65	1028
		Pseudomonas synxantha	1028
4	AM	*Streptococcus agalactiae*	624
		Streptococcus agalactiae	600
	BQ	*Actinomyces viscosus*	997
		Actinomyces viscosus	908

spectrum of bacteria identified may not completely mimic the urethral flora in situ. Third, definite proof for the association of the bacteria identified with the urethral epithelium is not available, i.e., the bacteria could also originate from the bladder or other anatomical locations.

Despite these pitfalls, we successfully documented the cure of a *M. genitalium* infection in one of the subjects, which serves as an excellent technological process control. Recent data by Bjornelius et al. (4) revealed that *M. genitalium* could be detected in more than 36% of all patients. It was also shown that in 30% of all cases of chronic nongonococcal urethritis, *U. urealyticum* could be detected (14), although the general literature is inconclusive with respect to the importance of this bacterial species (14, 24, 27). We did not find evidence for the presence of infectious *U. urealyticum* among our acute NCNGU patients. In addition, various species of *Haemophilus* have been implicated in NCNGU (27). Interestingly, patient 3 of the present study seemed to be harboring various species of this particular genus as well. *Haemophilus* spp. were not encountered among the healthy controls. Finally, the presence of various species of oral streptococci (*Streptococcus gordoni*, an unidentified oral bacterium AP60-15, and *Streptococcus salivarius* in patients 2, 3, and 5, respectively) may corroborate an earlier suggestion concerning the involvement of oral sex in the pathogenesis of NCNGU (13).

For some species, involvement in NCNGU was denounced on the basis of previous studies performed by others (9, 32). On the other hand, many other species have been previously indicated as possibly involved in the establishment of NCNGU. Whether these candidates, including, for instance, *U. urealyti-*

FIG. 6. Phylogram based on comparative analysis of the ribosomal sequences obtained for the clones derived from urine sediments of patients and controls. The tree was constructed from a weighted residue weight table. Clones B and D are the types that are most prevalent among both patients and controls (present in P1 to P5 and C1 to C5). The entries derived from the patients' clones are identified by patient numbers (see also Table 4). The clones are identified to a putative species level on the basis of BLAST searching. Clusters harboring streptococci, the *M. genitalium* sequences, or the *Haemophilus* and *Pseudomonas* spp.-like sequences are obvious. The scale at the bottom runs from 0 to 100% homology.

cum and *Gardnerella vaginalis* (9, 19), should be excluded from future studies cannot be decided on the basis of the present study, since the number of patients was kept low because of the experimental complexity of our in vitro work. We did not identify obvious and novel, putatively pathogenic bacterial species that are 100% associated with acute NCNGU. Although the RFLP analyses identified numerous types that were confined to pretreatment patient samples, DNA sequencing revealed that not a single bacterial species was exclusively present in all five disease-related, pretreatment samples and absent in all control samples. However, several uncharacterized bacterial species were identified more often (for instance, the DJAT 434 clone was detected in four of five patients versus one of five control subjects). In addition, Fig. 6 highlights the fact that, among the patients, *Pseudomonas*-like bacterial species were identified relatively frequently. The prevalence in NCNGU and the pathogenic potential of these bacterial species need to be defined more precisely. This requires the development of diagnostic tests for these elusive microorganisms that allow larger groups of patients to be screened. The reverse situation was documented once: RFLP type H occurred in all of the controls and in none of the patients. This difference, even with the limited number of individuals included in the current study, is statistically significant. The bacterial species most homologous to the RFLP type H DNA sequence appeared to have been detected in fresh lake water (12). Whether this bacterial species acts as a putative probiotic or whether it is simply outcompeted by the pathogens involved remains to be elucidated, just as diagnostic tests for this species need to be developed in order to more accurately determine its prevalence in healthy and diseased male urethras.

The major findings presented here are, first, the significant inter- and intrapersonal variability of the urethral flora, both in healthy and infected individuals, although this conclusion may be biased by the fact that only 50 clones were analyzed per urine sample. Second, azithromycin treatment seems to have little effect on the variability, complexity, and dynamics of the resident flora: many species not only disappear or appear during antibiotic treatment but also in the healthy, untreated situation. It is reassuring to see that in two of five patients, previously suggested pathogens were encountered that disappeared upon antibiotic treatment. The detection of a diversity of uncharacterized *Pseudomonas*-like bacterial species suggests that there is much more to explore in the bacterial flora of the male urethra, whereas the detection of bacterial species that disappear upon disease development may have important future implications to the therapy of NCNGU.

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