Specific *Neisseria gonorrhoeae* DNA-Probes Derived from Ribosomal RNA

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(Received 11 November 1988; revised 16 January 1989; accepted 26 January 1989)

Eighteen sequences complementary to less-conserved regions within the 16S and 23S ribosomal ribonucleic acid (rRNA) of *Neisseria gonorrhoeae* were subcloned or chemically synthesized and used as probes in a dot-spot deoxyribonucleic acid (DNA): DNA hybridization format. Some of these probes exclusively detected *Neisseria gonorrhoeae* nucleic acid, whereas others also showed hybridization signals with nucleic acid from other bacterial species. Our results indicate that rRNA-derived DNA-probes can be used to differentiate between very closely related taxa without the use of Southern-blot analysis.

INTRODUCTION

The literature dealing with nucleic acid hybridization probes for detection and identification of micro-organisms is accumulating very rapidly (Tenover, 1988). Most of these probes, which can be either total genomic deoxyribonucleic acid (DNA), indigenous plasmids, cloned DNA fragments or synthetic oligonucleotides, target the DNA of the organism to be detected. Because of the high abundance of ribosomes in a cell, it is obvious that probes targeting the ribosomal ribonucleic acid (rRNA), can considerably increase the sensitivity of a diagnostic test. Another advantage of rRNA-derived probes lies in the single-strandedness and ubiquity of the rRNA. On the other hand, the high sequence conservation observed among rRNA cistrons of different species is considered to be an important drawback of the method. Up to now, rRNA-derived probes have been used mainly to detect large groups of organisms such as *Legionella* (Wilkinson *et al.*, 1986; Edelstein, 1986), the *Pseudomonas fluorescens* group (Festl *et al.*, 1986), or to differentiate between relatively distantly related species such as *Mycoplasma* (Göbel *et al.*, 1987) and *Chlamydia* species (Palmer *et al.*, 1986). One report describes the differentiation between the species *Proteus vulgaris* and *Proteus mirabilis* (Haun & Göbel, 1987). These species have a DNA homology value of about 50%; currently, this is the highest degree of discrimination which can be achieved using rRNA-derived probes without the use of Southern hybridizations.

In the present study, the feasibility of using rRNA-derived DNA-probes to differentiate between highly related taxa was investigated. *Neisseria gonorrhoeae*, *Neisseria meningitidis* and related taxa were chosen as test organisms because the genotypic relationships of these taxa are amply documented (e.g. Guibourdenche *et al.*, 1986; R. Rossau and others, unpublished results), and some of the species are of high clinical relevance. In spite of the high sequence conservation of rRNA cistrons, our results prove that differentiation at the species level and even at the subspecies level can be achieved with rRNA-derived DNA-probes without the use of Southern-blot analysis.

Abbreviations: HT, hybridization temperature; WT, wash temperature; FA, formamide; PB, phosphate buffer.

Table 1. *Strains used*

Strain no.	Name	Culture collection no.*	Strain no.	Name	Culture collection no.*
1	<i>Neisseria gonorrhoeae</i>	NCTC 8375 ^T	32	<i>Neisseria subflava</i>	ITG 3821
2	<i>Neisseria gonorrhoeae</i>	ITG 4339	33	<i>Neisseria sicca</i>	ITG 3383
3	<i>Neisseria gonorrhoeae</i>	ITG 4085	34	<i>Neisseria elongata</i> subsp. <i>elongata</i>	NCTC 10660 ^T
4	<i>Neisseria gonorrhoeae</i>	ITG 4308	35	<i>Neisseria elongata</i> subsp. <i>glycolytica</i>	NCTC 11050
5	<i>Neisseria gonorrhoeae</i>	ITG 3939	36	<i>Neisseria canis</i>	ATCC 14687 ^T
6	<i>Neisseria gonorrhoeae</i>	ITG 4363	37	<i>Neisseria animalis</i>	NCTC 10212 ^T
7	<i>Neisseria gonorrhoeae</i>	ITG 4367	38	<i>Neisseria denitrificans</i>	ATCC 14686 ^T
8	<i>Neisseria gonorrhoeae</i>	ITG 4401	39	CDC group M-5	CCUG 4007
9	<i>Neisseria gonorrhoeae</i>	ITG 4437	40	CDC group EF-4a	CDC T-191/78
10	<i>Neisseria meningitidis</i>	NCTC 10025 ^T	41	<i>Kingella denitrificans</i>	NCTC 10995 ^T
11	<i>Neisseria meningitidis</i>	ITG 3342	42	<i>Kingella denitrificans</i>	NCTC 10997
12	<i>Neisseria meningitidis</i>	ITG 3343	43	<i>Kingella kingae</i>	NCTC 10746
13	<i>Neisseria meningitidis</i>	ITG 3345	44	<i>Simonsiella muelleri</i>	ATCC 29452
14	<i>Neisseria meningitidis</i>	ITG 3346	45	<i>Simonsiella crassa</i>	ATCC 15533 ^T
15	<i>Neisseria meningitidis</i>	ITG 3348	46	<i>Simonsiella steedae</i>	ATCC 27398
16	<i>Neisseria meningitidis</i>	ITG 3349	47	<i>Simonsiella species</i>	ATCC 27381
17	<i>Neisseria meningitidis</i>	ITG 3350	48	<i>Alysiella filiformis</i>	CCUG 3710 ^T
18	<i>Neisseria meningitidis</i>	ITG 3357	49	<i>Eikenella corrodens</i>	NCTC 10596 ^T
19	<i>Neisseria meningitidis</i>	ITG 3362	50	<i>Eikenella corrodens</i>	HIM 801-1
20	<i>Neisseria polysaccharea</i>	CIP N462 ^T	51	<i>Chromobacterium violaceum</i>	NCTC 9757 ^T
21	<i>Neisseria lactamica</i>	NCTC 10616	52	<i>Chromobacterium fluviatile</i>	LMG 6574
22	<i>Neisseria lactamica</i>	NCTC 10617 ^T	53	<i>Aquaspirillum dispar</i>	ATCC 27650
23	<i>Neisseria lactamica</i>	ITG 3689	54	<i>Pseudomonas testosteroni</i>	ATCC 17407
24	<i>Neisseria lactamica</i>	ITG 3690	55	<i>Oligella urethralis</i>	LMG 6227
25	<i>Neisseria cinerea</i>	NCTC 10294	56	<i>Haemophilus ducreyi</i>	CIP 542 ^T
26	<i>Neisseria mucosa</i>	CIP 59.51 ^T	57	<i>Kingella indologenes</i>	NCTC 10717 ^T
27	<i>Neisseria mucosa</i>	CIP 59.48	58	<i>Moraxella (Branhamella) catarrhalis</i>	NCTC 4103
28	<i>Neisseria mucosa</i>	CIP 59.47	59	<i>Escherichia coli</i>	B
29	<i>Neisseria macacae</i>	CIP N4176 ^T			
30	<i>Neisseria flavescens</i>	ATCC 13120 ^T			
31	<i>Neisseria subflava</i>	ATCC 10555			

* ATCC, American Type Culture Collection, Rockville, Md., USA; CCUG, Culture Collection of the University of Göteborg, Department of Clinical Bacteriology, University of Göteborg, Sweden; CDC, Centers for Disease Control, Atlanta, Ga., USA; CIP, Collection de l'Institut Pasteur, Paris, France; HIM, Hygiene-Institut, Marburg, FRG; ITG, Instituut voor Tropische Geneeskunde, Antwerp, Belgium; LMG, Laboratorium voor Microbiologie, Rijksuniversiteit, Gent, Belgium; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, UK: T, type strain.

METHODS

Strains used. The strains used are listed in Table 1. The identity of the strains was checked by conventional methods.

DNA preparations. High-molecular-mass genomic DNA was prepared essentially by the method described by Marmur (1961). Plasmid DNA was isolated by the method described by Kahn *et al.* (1979) and purified by CsCl-gradient centrifugation.

Fixation of denatured DNA on nitrocellulose membranes. The DNA solution (approximately 25 ng DNA μl^{-1} in 10 mM-Tris/HCl, pH 7.6, 1 mM-EDTA) was heated for 10 min at 95 °C, put on ice, and adjusted to 6 $\times$ SSC (1 $\times$ SSC is 0.15 M-NaCl, 0.015 M-sodium citrate, pH 7.0). The appropriate amount of solution was applied to a BA85 membrane (Schleicher and Schuell) in a dot-spot manifold (Bio-Rad). After air-drying, the membrane was baked at 80 °C for 2 h.

Construction of the probes 3 and 6. The plasmids pNGD1 and pNGK3 contain probes 3 and 6, respectively, as inserts. They were constructed from pNG4 and pNG3 respectively, which are recombinant plasmids containing part of the 16S rRNA gene of *N. gonorrhoeae* NCTC 8375^T cloned in pTZ18R (Pharmacia) (R. Rossau, unpublished results). In the case of pNGD1, a 71 bp *Sst*I-*Kpn*I fragment was subcloned and 25 bp beginning from the *Sst*I site were subsequently removed by Exonuclease III (Stratagene) and mung bean nuclease (Stratagene) treatment. For the construction of pNGK3, 63 bp were similarly removed from the 3' end of the insert of pNG3.

The resulting plasmid was cleaved with restriction enzymes *Sph*I and *Bst*XI, followed by blunting of the sticky ends using Klenow enzyme and intramolecular ligation. The restriction enzymes were purchased from Boehringer Mannheim or BioLabs and used as recommended by the suppliers.

Oligonucleotide synthesis and purification. The oligonucleotides were synthesized by the phosphite-triester method on a Gene Assembler 18-5800-01 (Pharmacia) or a Cyclone 8400 (New Brunswick). The deprotected oligonucleotides were purified on a 15% (w/v) polyacrylamide gel in 7 M-urea. The gel slice containing the oligonucleotide was chopped in pieces and the oligonucleotide was eluted overnight and desalted on a Sephadex G-25 (Pharmacia) column as described by Maniatis *et al.* (1982).

Probe labelling. The synthetic oligonucleotides used as probes were labelled using T4-polynucleotide kinase (Pharmacia) and [γ - 32 P]ATP (Amersham) (Maniatis *et al.*, 1982). To eliminate interference due to vector sequences during hybridization, the inserts of pNGD1 (probe 3) and pNGK3 (probe 6) were cut-out using restriction enzymes and purified by agarose gel electrophoresis. The purified inserts were labelled by filling in the sticky-ends with [α - 32 P]dATP (Amersham) using Klenow enzyme (Boehringer Mannheim) (Maniatis *et al.*, 1982). Unincorporated label was removed using a Bio-Gel P-6DG (Bio-Rad) spun-column (Maniatis *et al.*, 1982).

Hybridizations. A similar hybridization protocol was followed in all experiments except that conditions were adapted to the nature of the probes used. Prehybridization was usually done in plastic bags in $3 \times$ SSC, 25 mM-sodium phosphate buffer, pH 7.1 (PB), deionized formamide (FA) (20%, v/v), Ficoll (0.02%, w/v), bovine serum albumin (0.02%, w/v), polyvinylpyrrolidone (0.02%, w/v) and sheared, denatured salmon sperm DNA (0.1 mg ml^{-1}) at the temperature of hybridization for periods from 30 min to 4 h. Hybridization was allowed to proceed for periods from 1 h to overnight in the same solution to which approximately 0.5 to 1×10^6 c.p.m. ml^{-1} 32 P-labelled probe was added. The hybridization temperature (HT) depended on the experimental requirements. Following a brief rinse in $3 \times$ SSC, 25 mM-PB, 20% (v/v) FA at room temperature, the membranes were washed for 15 to 30 min in $3 \times$ SSC, 25 mM-PB, 20% (v/v) FA at the wash temperature (WT) indicated in the figures. Afterwards, the membranes were rinsed in $1.5 \times$ SSC at room temperature for approximately 10 min, dried and autoradiographed.

Table 2. *Probes used, their allocation on the rRNA molecule, length, percentage sequence homology with the corresponding sequences in the rRNA molecules of P. testosteroni and E. coli, and hybridization results with DNA from the type strains of N. gonorrhoeae and N. meningitidis*

Abbreviations: EC, *E. coli*; PT, *P. testosteroni*; NG^T, *N. gonorrhoeae* (type strain); NM^T, *N. meningitidis* (type strain).

Probe	rRNA	Region (in Fig. 1)	Length (bases)	Percentage homology		Hybridization with DNA from:*	
				PT	EC	NG ^T	NM ^T
1	16S	III	27	67	37	+	—
2	16S	III	37	64	46	+	—
3	16S	III	47	72	55	+	—
4	16S	III	57	72	56	+	—
5	16S	V	34	53	44	+	—
6	16S	VII	56	75	70	+	+
7	23S	VII	27	—	54	+	+
8	23S	VII	28	—	57	+	+
9	16S	I	32	38	63	+	—
10	23S	VI	39	—	40	+	+
11	23S	I	33	—	48	+	—
12	16S	IV	28	54	46	+	+
13	16S	II	24	58	42	+	—
14	16S	VI	26	50	46	+	—
15	23S	II	21	—	52	+	+
16	23S	III	28	—	43	+	—
17	23S	V	30	—	60	+	—
18	23S	IV	28	—	46	+	+

* Under stringent conditions, i.e. wash temperature approximately 5 °C below the dissociation temperature versus NG^T DNA.

Fig. 1. Allocation of the regions from which DNA-probes were constructed on the presumptive 16S rRNA secondary structure of *N. gonorrhoeae* (R. Rossau and others, unpublished results). These regions are indicated by thick lines and are identified by a Roman numeral.

RESULTS AND DISCUSSION

Probes used

In order to select *N. gonorrhoeae*-specific probes, one of the rRNA cistrons of the type strain of *N. gonorrhoeae* was cloned and sequenced (Rossau *et al.*, 1988; unpublished results). Evolutionarily less-conserved regions within the cistron were identified by alignment with known sequences (Dams *et al.*, 1988). Some of these regions were subcloned (probes 3 and 6) or chemically synthesized (probes 1, 2, 4, 5 and 7 to 18) and used as hybridization probes (Table 2). The locations of the probes derived from the 16S rRNA gene which were used in this study are shown on the diagram in Fig. 1. Fig. 2 shows the allocation of the 23S rRNA-derived probes on the secondary structure of the 23S rRNA molecule of *Escherichia coli*. From region III (in Fig. 1) of the 16S rRNA, four probes with different lengths (27, 37, 47 and 57 bases for probes 1, 2, 3 and 4, respectively) were tested. Probes 7 and 8 were derived from the same region of the 23S rRNA (region VII, Fig. 2). The sequence of probe 8 is identical to the sequence of probe 7,

Fig. 2. Allocation of the regions from which DNA-probes were constructed on the 23S rRNA secondary structure of *E. coli* (Noller, 1984). The regions are indicated by thick lines and are identified by a Roman numeral.

except that in probe 8 the adenosine residue at position 13 was deleted. Only one probe was used from all other regions.

Specificity of the probes

The criterion for specificity was the ability to differentiate between the *N. gonorrhoeae* and *N. meningitidis* type strains. Several independent studies (Kingsbury, 1967; Elwell & Falkow, 1977; Hoke & Vedros, 1982; Riou *et al.*, 1983; Guibourdenche *et al.*, 1986; R. Rossau and others, unpublished results) have shown that, despite their distinct pathogenic character, both species are genotypically extremely highly related. The DNA:DNA hybridization homology values reported between representatives of both species range between 64 and 93%. Using the initial renaturation rate method (De Ley *et al.*, 1970), the degree of binding between the type strains of both species was 74% (mean of three measurements; R. Rossau and others, unpublished results). These values are most often found among members of the same species. Species and subspecies are extremely ambiguous concepts. Recently, a proposal was put forward stating that 'a species generally would include strains with approximately 70% or greater DNA-DNA relatedness and with 5 °C or less ΔT_m ' (Wayne *et al.*, 1987).

In a preliminary series of experiments, the probes were hybridized with dot-spotted, denatured, genomic DNA of *N. gonorrhoeae* NCTC 8375^T, *N. meningitidis* NCTC 10025^T and *E. coli* B. From the autoradiographs in Fig. 3, it is clear that all probes derived from regions I, II, III, V, and VI (Fig. 1) of the 16S rRNA and probes derived from regions I, III, and V of the 23S rRNA, are specific for the type strain of *N. gonorrhoeae* at the appropriate wash temperature (see also Table 2). This is not the case for probes derived from regions IV and VII of 16S rRNA, or for probes derived from regions II, IV, VI and VII of 23S rRNA.

Probes 1, 5 and 9 were hybridized with genomic DNA from nine *N. gonorrhoeae* strains (strains 1 to 9 in Table 1) and ten *N. meningitidis* strains (strains 10 to 19 in Table 1). As shown in

Fig. 3. Specificity of the probes at different wash-temperatures. Following hybridization at the temperature (HT) and with the probe indicated, the membranes, on which 1 µg of denatured DNA of *N. gonorrhoeae* NCTC 8375^T (NG), *N. meningitidis* NCTC 10025^T (NM) and *E. coli* B (EC) was spotted, were washed for 15 min at the indicated wash-temperature (WT), dried and autoradiographed for 24 h with an intensifying screen at -70 °C.

Fig. 4. Differentiation between *N. gonorrhoeae* and *N. meningitidis* strains using a dot-spot DNA :DNA hybridization procedure. Denatured DNA (1 µg) from nine *N. gonorrhoeae* strains (rows A, B and C, 1 to 3), ten *N. meningitidis* strains (rows D, E and F, 1 to 3, and G1), and one strain each of *P. testosteroni* (G2) and *E. coli* (G3) were spotted onto nitrocellulose, hybridized with probe 1 (panel A), probe 5 (panel B) and probe 9 (panel C) at 50, 55 and 60 °C respectively, and washed at the temperatures indicated.

Fig. 4, these probes duplexed with the DNA from all *N. gonorrhoeae* strains tested. Probes 5 and 9 were specific for *N. gonorrhoeae* strains at high stringencies only, whereas probe 1 remained specific at low-stringency conditions.

The possibility of non-specific cross-hybridization of some of the probes was investigated by hybridizations with purified genomic DNA from a variety of bacteria, the genotypical relationships of which are shown in Fig. 5. Probes 3, 5 and 7 were hybridized with genomic DNA from seven *Neisseria* species and some other Gram-negative bacteria (Fig. 6). Under the conditions used, probes 3 and 5 did not form stable duplexes with any of the DNAs tested, except for *N. gonorrhoeae* DNA. Even the less-specific probe 6 did not produce a detectable signal with non-neisserial DNA. The highly specific probe 1 and the less-specific probe 10 were hybridized with purified DNA from 44 strains belonging to bacterial taxa which are highly or moderately related to *N. gonorrhoeae* in terms of rRNA cistron similarities (strains 1, 7, 10, 17, and 20 to 50 in Table 1) or taxa which are more distantly related (strains 51 to 59 in Table 1; see also Fig. 5). As shown in Fig. 7, probe 1 hybridizes exclusively with DNA from the *N. gonorrhoeae* strains. In contrast, probe 10 hybridizes with DNA from almost all *Neisseria* species

Fig. 5. Diagram representing the genotypical relationships of the organisms used. On the axis the genotypical relationships of the organisms are expressed in terms of rRNA similarities ($T_{m(e)}$ in °C) versus reference rRNA of *N. flavescens* ATCC 13120^T (R. Rossau and others, unpublished results). The $T_{m(e)}$ is the temperature at which 50% of a DNA:rRNA hybrid is denatured (De Ley & De Smedt, 1975). The higher the $T_{m(e)}$, the closer the organism is related to the reference organism (in this case *N. flavescens*). The dendrogram shows the genotypical relationships of the organisms found in the uppermost part of the $T_{m(e)}$ -axis (*Neisseria* and relatives, group I) as deduced from DNA:DNA hybridization data and expressed in degree of binding (%D; De Ley *et al.*, 1970). All other organisms do not exhibit a significant %D with the taxa belonging to the *Neisseria* and relatives group I (R. Rossau and others, unpublished results). The phylogenetic relationships of *N. cinerea* and *N. polysaccharea* were deduced from DNA:DNA hybridization data published by Guibourdenche *et al.* (1986); the position of these organisms is indicated relative to *N. gonorrhoeae* on the DNA:DNA dendrogram. The exact taxonomical position of *N. macacae* is not known.

and DNA from *Kingella kingae*, *Simonsiella muelleri*, *Simonsiella crassa* and *Alysiella filiformis*. No hybridization signal was observed with all other DNAs, including DNA from *Neisseria canis*, *Neisseria denitrificans* and *Neisseria animalis*.

From the results shown in Figs 3 and 4, it is obvious that the specificity of the probes is highly dependent on the hybridization and wash conditions used. For instance, by simply altering the wash temperature, the detection range of the probes can be extended, so that one and the same probe can be used to detect *N. gonorrhoeae* specifically, or a larger group of organisms, as illustrated for probe 3 in Fig. 8.

Some properties of the probes and the results obtained are summarized in Table 2. Eleven of the probes tested hybridized with DNA from the *N. gonorrhoeae* type strain but not with DNA from the *N. meningitidis* type strain. At least three of these probes could discriminate between

Fig. 6. Hybridization results of selected rRNA-derived DNA-probes with DNA from several representative Gram-negative bacteria. Denatured DNA (1 µg) from *N. gonorrhoeae* NCTC 8375^T (A1), *N. meningitidis* NCTC 10025^T (B1), *N. lactamica* NCTC 10617^T (C1), *N. mucosa* CIP 59.51^T (D1), *N. subflava* ATCC 10555 (E1), *N. flavescens* ATCC 13120^T (F1), *N. elongata* ssp. *elongata* NCTC 10660^T (A2), *Chromobacterium violaceum* NCTC 9757^{NT} (B2), *P. testosteroni* ATCC 17407 (C2), *Haemophilus ducreyi* CIP 542^T (D2), *Moraxella (Branhamella) catarrhalis* NCTC 4103 (E2) and *E. coli* B (F2), was spotted on nitrocellulose, hybridized at temperature HT with the probe indicated and washed at the temperature WT.

Fig. 7. Hybridization of probe 1 and probe 10 with DNA from a variety of Gram-negative bacteria. From each strain (the numbers correspond to the numbering in Table 1) denatured DNA (1 µg) was applied to nitrocellulose and hybridized and washed at the indicated temperatures.

different randomly chosen *N. gonorrhoeae* and *N. meningitidis* strains (see Fig. 4). Table 2 illustrates that there is a broad fluctuation in sequence homology values (from 37 to 75%). However, there is no absolute correlation between the percentage sequence homology and the specificity of the probes; probes from regions with low homology values are not always specific and *vice versa*. This might be due to differences in the mode or rate of evolution in different phyla and in the different regions of the rRNA molecules.

The results presented here indicate that there are small but consistent sequence differences in certain regions of the rRNA molecules of highly related taxa, which can be detected by a hybridization assay. This observation has some widespread implications for the use of rRNA-derived probes in general. rRNA-derived DNA-probes cannot be used exclusively to identify large groups of organisms, but can be used to differentiate between organisms at the species or subspecies level. Provided that the probe sequence and the hybridization conditions are

Fig. 8. Alteration of the detection range of a probe by changing the stringency of the wash conditions. Membranes, to which denatured DNA (1 µg) from *N. gonorrhoeae* NCTC 8375^T (NG), *N. meningitidis* NCTC 10025^T (NM), *N. elongata* ssp. *elongata* NCTC 10860^T (NE), *C. violaceum* NCTC 9575^{NT} (CV), *P. testosteroni* ATCC 17407 (PT) and *E. coli* B (EC) was fixed, were hybridized at 50 °C with ³²P-labelled probe 3 in the absence of formamide. Afterwards, the membranes were washed at 50, 60, 70 and 80 °C, respectively, in 3 × SSC and 25 mM-PB. The membranes were dried and autoradiographed overnight at -70 °C with an intensifying screen.

carefully chosen, highly specific identification can be accomplished in a simple dot-spot DNA:DNA hybridization format, making tedious Southern-blot analyses obsolete. In particular, some of the specific probes described in this report could find application in culture confirmation of *N. gonorrhoeae* and the diagnosis of *N. gonorrhoeae* in all types of clinical samples, since no interference is likely from other micro-organisms under appropriate hybridization conditions. Although highly specific probes are not an absolute prerequisite for this purpose, the probes described here can be used also for taxonomic or epidemiological investigations based on restriction fragment length polymorphism analysis (Grimont & Grimont, 1986; Stull *et al.*, 1988), or to identify and classify related but hitherto undescribed or unclassified micro-organisms.

We wish to thank Miss Martine Baudewijn for typing the manuscript.

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