The development of specific rRNA-derived oligonucleotide probes for *Haemophilus ducreyi*, the causative agent of chancroid

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Part of a ribosomal ribonucleic acid (rRNA) cistron of *Haemophilus ducreyi* was enzymically amplified using conserved primers within the rRNA molecules, cloned in a plasmid vector, and sequenced. From the nucleotide sequence, eight oligonucleotides complementary to different regions in the 16S and 23S rRNA molecules were selected, chemically synthesized, and used as hybridization probes. Hybridization experiments with at least 41 *H. ducreyi* strains and 13 or 14 non-*H. ducreyi* strains revealed that all eight oligonucleotide probes were highly reliable and completely specific for *H. ducreyi* strains. Comparisons of 16S rRNA sequences confirm that *H. ducreyi* is a member of the *Pasteurellaceae* though not closely related to other species in this family.

Introduction

Haemophilus ducreyi, the causative agent of chancroid, is a fastidious Gram-negative bacterium, assigned to the genus *Haemophilus* by Kilian (1976) on phenotypic grounds. Casin *et al.* (1985) and Albritton (1989) showed that *H. ducreyi* is a homogeneous species which lacks DNA relatedness to other *Haemophilus* species or to other members of the family *Pasteurellaceae*. Based on these and other observations, Albritton (1989) questioned the inclusion of *H. ducreyi* in the family *Pasteurellaceae*. A recent study on the taxonomy of the *Pasteurellaceae* using DNA:rRNA hybridization shows that *H. ducreyi* is a distinct taxon in the family *Pasteurellaceae* (De Ley *et al.*, 1990).

Although isolation and culture of *H. ducreyi* is difficult and the sensitivity of diagnosis by culture may be relatively poor, culture remains the method of choice for the diagnosis of chancroid (Ronald & Albritton, 1984). A recent approach to the diagnosis of infectious agents makes use of DNA-probe technology (Viscidi & Yolken, 1987; Tenover, 1988), which is particularly advantageous in the diagnosis of fastidious organisms. More recently the use of chromosomal DNA probes for the detection of growth-amplified *H. ducreyi* strains has been

reported (Parsons *et al.*, 1989). However, a DNA-probe assay for this micro-organism would be especially useful if direct detection in a clinical sample could be achieved. Due to the high sensitivity required, DNA probes targeting the abundant and single-stranded rRNA molecules are preferred to probes directed against genes located on the chromosome which, in general, are present in only one or a few copies. Earlier published results showed that despite the highly conserved character of the rRNA genes, high specificities could be attained when using short oligonucleotides complementary to certain regions in the rRNA molecules as hybridization probes (Haun & Göbel, 1987; Rossau *et al.*, 1989). Since *H. ducreyi* seems to be genotypically homogeneous and relatively distantly related to other known bacteria (Casin *et al.*, 1985; De Ley *et al.*, 1990), the development of a species-specific rRNA-derived DNA probe should be feasible. Therefore, a rapid cloning system for rRNA genes was developed making use of the polymerase chain reaction (PCR) and oligonucleotide primers allocated within the more conserved regions of the rRNA molecules. The 16S rRNA gene and a part of the 23S rRNA gene of *H. ducreyi* CIP 542^T were cloned and sequenced. Five 16S rRNA-derived oligonucleotides and three 23S rRNA-derived oligonucleotides were chemically synthesized and their specificity (i.e. the ability to differentiate *H. ducreyi* organisms from others) and reliability (i.e. the ability to identify all *H. ducreyi* isolates) were evaluated.

Abbreviation: PCR, polymerase chain reaction.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M38751-23SV.

Alignment and signature analysis of the rRNA sequences were used to elucidate the taxonomic position of *H. ducreyi*.

Methods

Strains and culturing methods. *H. ducreyi* strains originating from different locations in Africa (52 strains), Asia (9 strains), Europe (14 strains) and North America (12 strains), predominantly isolated from genital ulcers, were used. These, and the reference strains of other bacterial species used, are listed in Table 1. *H. ducreyi* cells, grown on Mueller-Hinton (BBL) agar supplemented with 5% (v/v) heated (80 °C) horse blood, 1% (v/v) Isovitalex (BBL), and 5% (v/v) foetal calf serum (Gibco) or on GC medium (Difco) agar supplemented with 1% (w/v) bovine haemoglobin (Difco), 1% (w/v) Isovitalex, and 5% (v/v) foetal calf serum (Gibco) at 33–35 °C in a CO₂, (5%, v/v) enriched atmosphere for 48 h, were supplied by P. Piot and collaborators (Institute of Tropical Medicine, Antwerp, Belgium).

DNA isolation. Whole-cell DNA was prepared essentially by the method of Marmur (1961). Recombinant plasmids were isolated using the method described by Kahn *et al.* (1979). For sequence analysis, plasmid DNA was purified using the Gene-Clean kit (BIO 101) as recommended by the manufacturers.

Preparation of the membranes. A few colonies of each isolate were scraped from the agar plate and applied to a Biotryne A transfer membrane (Pall Ultrafine Filtration Corp.). The cells were lysed by incubating the membranes at room temperature on Whatman 3 MM filter paper saturated with 10% (w/v) SDS. When lysis was apparent, usually after about 5 min, the membranes were dried and baked for 2 h at 80 °C. Purified whole-cell DNA was fixed to nitrocellulose membranes (BA 85, Schleicher & Schüll) as described previously (Rossau *et al.*, 1989).

Oligonucleotide synthesis, purification and labelling. The oligonucleotides to be used as primers or probes were synthesized by the phosphite-triester method on a Cyclone 8400 DNA synthesizer (New Brunswick Scientific). The protected oligonucleotides were purified on Nensorb Prep columns (DuPont, NEN Research Products) as recommended by the manufacturers. The oligonucleotides were 5'-labelled with [γ -³²P]ATP and T4 polynucleotide kinase (Pharmacia) (Maniatis *et al.*, 1982) or tailed at their 3' end with digoxigenin-11-dUTP (Boehringer Mannheim) and terminal transferase (Boehringer Mannheim) in 0.1 M-sodium cacodylate, pH 7.0, 1 mM-CoCl₂, 0.1 mM-dithiothreitol and 50 µg bovine serum albumin ml⁻¹ for 30 min at 37 °C.

Enzymic amplification. A 2400 bp fragment of a rRNA cistron of *H. ducreyi* CIP 542^T was enzymically amplified using the PCR technique, starting from 1 µg purified whole-cell DNA. The reagents supplied in the GeneAmp kit (Perkin-Elmer Cetus) were used according to the manufacturers' recommendations.

One hundred pmol of the following primers were added to a total reaction volume of 100 µl: TGGCTCAGAT TGAACGCTGG CGGC (primer 1) and CCTTCCCTC ACGGTACTGG T (primer 2). After initial denaturation at 95 °C for 4 min, 30 cycles were performed. Annealing was carried out for 2.5 min at 37 °C, extension for 3.5 min at 72 °C, and denaturation for 1.5 min at 95 °C. After 10 min incubation at 72 °C and cooling to room temperature, the mixture was extracted with 200 µl CHCl₃. The aqueous phase was then recovered and the nucleic acids precipitated overnight at –20 °C with 0.3 M-sodium acetate and 2 vols ethanol. The pellet was dissolved in 20 µl 10 mM-Tris/HCl (pH 7.6), 1 mM-EDTA (TE), loaded onto a 1% (w/v) agarose gel, and electrophoresed for about 90 min at 8 V cm⁻¹.

Table 1. Strains used

Taxon	Origin	No. of strains and/or collection no.*
<i>Haemophilus ducreyi</i>	South Africa	17
	Kenya	6
	Rwanda	23
	Zaire	6
	Thailand	7
	Sri Lanka	1
	Vietnam	1 (CIP 542 ^T)
	USA	3
	Canada	8
	Mexico	1
	The Netherlands	7
	Denmark	2
	France	3
	Belgium	2
	Unknown	2
<i>Escherichia coli</i>		2 (MC 1061 and B)
<i>Actinobacillus actinomyces</i>		NCTC 9710 ^T
<i>Actinobacillus lignieresii</i>		NCTC 4189 ^T
<i>Haemophilus aphrophilus</i>		NCTC 5906 ^T
<i>Haemophilus influenzae</i>		NCTC 8143 ^T
<i>Histophilus ovis</i>		HIM 896-7
<i>Pasteurella multocida</i>		NCTC 10322 ^T
<i>Moraxella catarrhalis</i>		ITM 4197
<i>Comamonas testosteroni</i>		ATCC 17407
<i>Oligella urethralis</i>		LMG 6227
<i>Neisseria gonorrhoeae</i>		ITM 4437
<i>Campylobacter jejuni</i>		CCUG 11284 ^T
<i>Acinetobacter calcoaceticus</i>		ATCC 23055 ^T

* ATCC, American Type Culture Collection, Rockville, MD, USA; CCUG, Culture Collection of the University of Göteborg, Department of Clinical Bacteriology, University of Göteborg, Sweden; CIP, Collection de l'Institut Pasteur, Paris, France; HIM, Hygiene-Institut, Marburg, FRG; ITM, Institute for Tropical Medicine, Antwerp, Belgium; LMG, Laboratorium voor Microbiologie, Rijksuniversiteit, Gent, Belgium; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, UK; ^T, type strain.

The gel portion containing the amplified DNA fragment of about 2400 bp was removed and the DNA eluted by centrifugation through an HVLP 025000 membrane filter (Millipore). The eluate was then extracted using phenol and ether, and the DNA precipitated with ethanol. The DNA was redissolved in 20 µl TE and used for restriction analysis and cloning.

Cloning. The gel-purified 2400 bp PCR fragment was treated with Klenow enzyme to create blunt ends as described by Maniatis *et al.* (1982), and the fragment was then further digested with *Xba*I. After overnight ligation at 15 °C with *Sma*I-*Xba*I-digested pSPT19 (Pharmacia), transformation in competent *Escherichia coli* MC1061 and overnight culture on Luria-Bertani agar containing ampicillin (0.1 mg ml⁻¹), clone analysis was performed on 16 colonies, as described by Birnboim & Doly (1979).

Nucleic acid sequence determination. The inserts of the recombinants were sequenced using the dideoxy chain-termination method (Sanger *et al.*, 1977) modified according to the protocol for double-stranded templates given in the reagent kit for DNA sequencing with Sequenase (United States Biochemical). Oligonucleotides complementary to sequences adjacent to the plasmid inserts or allocated in conserved

areas of the rRNA genes were used as primers. Sequence comparison was done using the PC/GENE software provided by Intelligenetics Inc. and Genofit SA.

Hybridizations. Biotinylated A membranes on which bacterial colonies were fixed were prehybridized in a mixture containing the following components: 3 × SSC (1 × SSC is 0.15 M-NaCl plus 0.015 M-sodium citrate, pH 7.0), 25 mM-sodium phosphate buffer (pH 7.1), 20% (v/v) deionized formamide, 0.02% Ficoll (type 400; Sigma), 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.1 mg sheared heat-denatured salmon sperm DNA ml⁻¹, and 0.2% SDS, usually for 0.5–1 h at the appropriate temperature. The hybridization mixture had the same composition except that approximately 10⁶ c.p.m. of ³²P-labelled probe ml⁻¹ was added. Hybridizations were performed at the same temperature for 1–2 h. The membranes were washed for 30 min in 3 × SSC, 25 mM-sodium phosphate buffer (pH 7.1), 20% (v/v) deionized formamide, 0.2% SDS at the hybridization temperature. Nitrocellulose membranes (BA 85) were processed similarly except that SDS was omitted from the mixtures. Fuji RX film (Fuji Photo Film) was used for autoradiography. Hybridizations with digoxigenin-labelled probes were performed as described in the protocol sheet of the DNA labelling and detection kit nonradioactive (Boehringer) except that 3 × SSC instead of 5 × SSC was used and that the mixture contained 20% (v/v) deionized formamide unless otherwise stated. About 1 pmol digoxigenin-labelled probe was used per ml hybridization mixture.

Results

Enzymic amplification and cloning

For the enzymic amplification, conserved primers allocated about 40 bp from the 5' end of the 16S rRNA gene and about 450 bp from the 5' end of the 23S rRNA gene were used (Fig. 1). As expected, enzymic amplification with these primers yielded a dominant product about 2400 bp in length and some minor fragments. The 2400 bp fragment was clearly visible on an ethidium-bromide-stained 1% agarose gel after electrophoresis of 10% of the total amplification reaction yield starting from 1 µg purified whole-cell DNA from *H. ducreyi* CIP 542^T (results not shown). Restriction endonuclease analysis of the purified 2400 bp PCR fragment revealed a unique restriction site for *Xba*I. Digestion of the amplified fragment with *Xba*I enabled the directional cloning of two *Xba*I blunt fragments of about 1600 bp and 800 bp, respectively, in the vector pSPT19 digested with *Sma*I and *Xba*I. Of the 16 clones analysed, one carried the 1600 bp fragment and six the 800 bp fragment. The resulting recombinant plasmids containing the 1600 bp and 800 bp fragments, respectively, were named pHDA1 and pHDA2 (Fig. 1). To facilitate the sequencing of the 16S and 23S rRNA genes, the subclones pHDA3, pHDA4, and pHDA6 were constructed by making use of the available restriction sites (see Fig. 1).

Nucleic acid sequence analysis

The sequencing reactions performed are indicated in Fig. 1. Dideoxy chain-termination sequencing on the purified plasmid DNA resulted in clearly readable sequencing ladders with only a few ambiguities. The nucleotide sequences of the 16S rRNA gene (except for about 40 nucleotides at the 5' end) and the first 456 nucleotides at the 5' end of the 23S rRNA gene are shown in Fig. 2(a) and Fig. 2(b), respectively. In the 16S and 23S rRNA genes all overlaps were obtained. The 3' end of the 16S rRNA gene and the 5' end of the 23S rRNA gene were determined by analogy with the *E. coli* sequences (Brosius *et al.*, 1981). The G + C content of the 16S rRNA gene was 51.7 mol%, while that of the 5' end of the 23S rRNA gene was 43.9 mol%. The base composition of the genome of *H. ducreyi* CIP 542^T is approximately 39 mol% G + C (Casin *et al.*, 1985; De Ley *et al.*, 1990).

The sequences were aligned with published sequences using the Fast scan program of PC/GENE and the percentage homology was calculated. The homology values obtained with partial 16S rRNA sequences of members of the *Pasteurellaceae* [regions A and B as described by Chuba *et al.* (1988)] and with 16S and 23S rRNA sequences of some other representative bacterial taxa are shown in Table 2. The homology values for region B and particularly for region A were lower than those obtained with virtually complete 16S rRNA sequences. However, when regions A and B were combined, all homology values with members of the *Pasteurellaceae* (86–93%) were higher than those obtained with other bacterial taxa (75–85%). From Table 2, it can also be inferred that the sequenced 5' part of the 23S rRNA was not highly conserved.

Ninety-two of the 95 16S rRNA signature nucleotides of the purple bacteria (now named *Proteobacteria*; Stackebrandt *et al.*, 1988) as defined by Woese (1987) could be deduced. The sequence signature of *H. ducreyi* CIP 542^T was highly similar to the sequence signature of gamma subdivision organisms. Only the following four of the 92 signature nucleotides compared differed from those of the gamma subdivision: (i) U instead of G at positions 718 and 760 in *H. ducreyi* (numbering as in Fig. 2) and *E. coli* (numbering as in Woese, 1987), respectively; (ii) A instead of G or U at positions 1216 and 1260, respectively; (iii) U instead of G at positions 1253 and 1297, respectively; and (iv) A instead of G at positions 1254 and 1298, respectively. Twenty-two signature positions between *H. ducreyi* and members of the *Pasteurellaceae* (Chuba *et al.*, 1988) could be compared and were found to be completely identical except that at position 390 (*E. coli* numbering), C instead of U was found in some species. It is striking, and most probably

Fig. 1. Enzymic amplification, cloning, subcloning, and sequencing scheme for a 2400 bp rRNA gene fragment of *H. ducreyi* CIP 542^T. The PCR fragment is indicated between black vertical arrowheads. The hatched area is the spacer region between the 16S and the 23S rRNA gene. The sequencing reactions are indicated by horizontal arrows. The locations of some relevant restriction sites are also indicated.

(a)

	10	20	30	40	50	60
1	GCAGGCTTAA	CACATGCAAG	TCGAACGGTA	GCACGAAGGA	CTTGCTCCTT	GGGTGACGAG
61	TGGCGGACGG	GTGAGTAATG	CTTGGGAATC	TGGCTTATGG	AGGGGGATAA	CTACGGGAAA
121	CTGTAGCTAA	TACCGCGTAA	TATCTTCGGA	TTAAAGGGTG	GGACCTTCGG	GCCACCTGCC
181	ATAAGATGAG	CCCAAGTGGG	ATTAGGTAGT	TGGTTAGGTA	AAGGCTGACC	AAGCCGACGA
241	TCTCTAGCTG	GTCTGAGAGG	ATGACCAGCC	ACACTGGAAC	TGAGACACGG	TCCAGACTCC
301	TACGGGAGGC	AGCAGTGGGG	AATATTGCAC	AATGGGGGAA	ACCCTGATGC	AGCCATGCCG
361	CGTGAATGAA	GAAGGCCCTC	GGGTTGTAAA	GTTCTTTTCG	TGATGAGGAA	GGCAGTTTGT
421	TTAATAGCAA	AATGATTGGA	CGTTAGTCAC	AGAAGAAGCA	CCGGCTAACT	CCGTGCCAGC
481	AGCCGCGGTA	ATACGGGGGG	TGCGAGCGTT	AATCGGAATA	ACTGGGCGTA	AAGGGCACGC
541	AGGCGGTTGA	TTAAGTGAGA	TGTGAAAGCC	CCGGGCTTAA	CCTGGGAATT	GCATTTTCATA
601	CTGGTCAACT	AGAGTACTTT	AGGGAGGGGT	AGAATTCAC	GTGTAGCGGT	GAAATGCGTA
661	GAGATGTGGA	GGAATACCGA	AGGCGAAGGC	AGCCCTTGG	GAATGTATNG	ANNCTCATGT
721	CGGAAAGCGT	GGGAGCAAAA	CAGGATTAGA	TACCTTGTTA	GTCCACGCTG	TAAACGATGT
781	CGATTGCGG	GTTGGTCTTT	AAGACTGGCG	CCCGAAGCTA	ACGTGATAAA	TCGACCGCCT
841	GGGAGTACG	GCCGCAAGGT	TAAACTCAA	ATGAATTGAC	GGGGCCCGC	ACAAGCGGTG
901	GAGCATGTGA	TTTAATTGCA	TGCAACGCGA	AGAACCTTAC	CTACTCTTGA	CATCCATAGA
961	AGAACTCAGA	GATGAGTTTG	TGCCTTCGGG	AACTATGTGA	CAGGTGCTGC	ATGGCTGTCG
1021	TCAGCTCGTG	TTGTGAAATG	TTGGGTAAAG	TCCCGCAACG	AGCGCAACCC	TTATCCTTTG
1081	TTGCCAGCAT	GTAATGATGG	AACTCAAAGG	AGACTGCCAG	TGATAAACTG	GAGGAAGGTG
1141	GGGATGACGT	CAAGTCATCA	TGGCCCTTAC	GAGTAGGGCT	ACACACGTGC	TACAATGGCG
1201	TATACAGAGG	GCGGCAAAAC	TGCAAAAGGG	AGCGAATCTC	ACAAAGTACG	TCTAAGTCCG
1261	GATTGGAGTC	TGCAACTCGA	CTCCATGAAG	TCGGAATCGC	TAGTAATCGC	AAATCAGAAT
1321	GTTGCGGTGA	ATACGTTCCC	GGGCCCTGTA	CACACCGCCC	GTACACCAT	GGGAGTGGGT
1381	TGTACAGAA	GTAGATAGCT	TAACCTTCGG	GAGGGCGTTT	ACCACGGTAT	GATTTCATGAC
1441	TGGGGTGAAG	TCGTAACAAG	GTAACCGTAG	GGGAACCTGC	GGTTGGATCA	CCTCCTTA

(b)

	10	20	30	40	50	60
1	AGTTAAGTGA	CTAAGCGTAC	ACGGTGGATG	CCTTGGCAAT	CAGAGGCGAT	GAAGGACGTG
61	CTAATCTGCG	ATAAGCTTGG	ATGAGTTGAT	CAAGAAACGT	ATAATCCAAG	ATCTCCGAAT
121	GGGGAAACCC	ACTAGATGAT	GAATCTAGTA	TTTATTACCC	AATACATAAG	TAATAAAAGC
181	AAACCGGGAG	AAGTGAACA	TCTAAGTAAC	CCGAGGAAAA	GAAATCAACC	GAGATTCCGT
241	TAGTAGCGGC	GAGCGAACGT	GGAAGGCGCA	GTTAGTGATA	ACCCATACAT	TAGAGGAAGT
301	AGCTGGGAAG	CTACATCATA	GAGGGAGATA	ATCCCGTACT	CGAAAATATG	TGGGCCGTAC
361	TAAACTAACA	ACAAGTAAGG	CGGGACACGA	GAAATCTGT	CTGAAGATGG	GNNNACCATC
421	CTCAAGGCT	AAATACTCCT	GATTGACCGA	TAGTGA		

Fig. 2. Nucleotide sequences of the non-coding strand of the 16S rRNA gene (a) and the 5' end of the 23S rRNA gene (b) of *H. ducreyi* CIP 542^T. Approximately 40 bp at the 5' end of the 16S rRNA gene could not be determined. N refers to nucleotides which could not be determined unambiguously.

Table 2. Percentage sequence homology of the rRNA sequences of *Haemophilus ducreyi* CIP 542^T with those of other bacterial taxa

In columns A and B the percentage homology of the regions in the 16S rRNA molecule for which sequences of members of the *Pasteurellaceae* are published (A and B, respectively, in Chuba *et al.*, 1988) is given. The total homology of region A plus B is shown in column A + B. The percentage homology with the virtually complete 16S rRNA sequence and the 5' part of the 23S rRNA sequence is given in columns 16S and 23S, respectively. The percentage homology was calculated by dividing the total number of matches by the total number of nucleotides compared. The total number of nucleotides used in the calculation is indicated between parentheses.

Source of the sequences: a, Chuba *et al.* (1988); b, Brosius *et al.* (1981); c, Carbon *et al.* (1981); d, unpublished results (R. Rossau and others); e, Toschka *et al.* (1987, 1988); f, Rossau *et al.* (1988 and unpublished results); g, Dorsch *et al.* (1989).

	Percentage sequence homology				
	A (100)	B (345)	A + B (445)	16S (1489)	23S (456)
<i>Actinobacillus hominis</i> ^a	83	96	93	—	—
<i>Actinobacillus equuli</i> ^a	79	95	91	—	—
<i>Actinobacillus lignieresii</i> ^a	80	93	90	—	—
<i>Haemophilus influenzae</i> ^a	82	93	90	—	—
<i>Actinobacillus actinomycetemcomitans</i> ^a	88	90	89	—	—
<i>Haemophilus aphrophilus</i> ^a	82	89	87	—	—
<i>Pasteurella multocida</i> ^a	84	87	86	—	—
<i>Escherichia coli</i> ^b	81	86	85	89	77
<i>Proteus vulgaris</i> ^c	78	86	84	87	—
<i>Moraxella catarrhalis</i> ^d	78	83	82	84	67
<i>Pseudomonas aeruginosa</i> ^e	76	79	78	83	75
<i>Neisseria gonorrhoeae</i> ^f	78	80	79	81	76
<i>Brucella abortus</i> ^g	72	75	75	78	—

not without significance, that U instead of G is also found in position 760 (*E. coli* numbering) in the 16S rRNA sequences of all members of the *Pasteurellaceae* published so far (Chuba *et al.*, 1988). The nucleotides in positions 1260, 1297, and 1298 (*E. coli* numbering) were not comparable.

The secondary structures of the 16S rRNA and the 5' end of the 23S rRNA were essentially identical to the consensus structures respectively proposed by Dams *et al.* (1988) and by both Gutell & Fox (1988) and Höpfel *et al.* (1989). The higher-order structure of the region between positions 180 and 220 (*E. coli* numbering) of the 16S rRNA of *H. ducreyi* is identical to that of beta and gamma subdivision organisms (Woese, 1987).

Probe specificity and reliability

From the available sequence information, subsequences allocated in variable regions of the 16S and 23S rRNA molecules (as described in Rossau *et al.*, 1989) were

Fig. 3. Specificity and reliability of digoxigenin-labelled probe 8 at different wash conditions. One microgram of dot-spotted denatured genomic DNA was hybridized and washed at 40 or 65 °C. At 65 °C the hybridization and wash mixtures did not include formamide; this corresponds to about 50 °C in mixtures with 20% (v/v) formamide. Row A, and B1 and B2, *Haemophilus ducreyi* strains; B3, *Escherichia coli*; C1, *Actinobacillus actinomycetemcomitans*; C2, *Actinobacillus lignieresii*; C3, *Haemophilus aphrophilus*; D1, *Haemophilus influenzae*; D2, *Histophilus ovis*; D3, *Pasteurella multocida*; E1, *Moraxella catarrhalis*; E2, *Comamonas testosteroni*; E3, *Oligella urethralis*; F1, *Neisseria gonorrhoeae*; F2, *Campylobacter jejuni*; F3, *Acinetobacter calcoaceticus*.

selected. The target regions of probes 2 and 3 were compared with published sequences of other members of the *Pasteurellaceae*; at least four mismatches were found. In the target regions of all the probes tested at least six mismatches were observed with organisms not belonging to the *Pasteurellaceae*. Oligonucleotides varying from 20 to 28 nucleotides in length and complementary to these selected sequences were chemically synthesized, labelled and used as hybridization probes.

The probes were hybridized at different temperatures with dot-spotted whole-cell DNA of five *H. ducreyi* strains and several strains of other bacterial taxa including six strains belonging to different species in the family *Pasteurellaceae*. At stringent hybridization and wash temperatures (i.e. about 5 °C below the experimental dissociation temperature, T_d , of the probe-target hybrid), none of the probes cross-hybridized with non-*H. ducreyi* strains. This is illustrated in Figs 3 and 4(a) for probes 8 and 7, respectively. The results obtained with all probes tested are summarized in Table 3.

Between 41 and 89 *H. ducreyi* strains isolated in different continents were screened at the hybridization and wash temperatures at which the probes were specific for *H. ducreyi* (Table 3). Either dot-spotted purified whole-cell DNA or colonies directly applied to nylon

Fig. 4. Specificity and reliability of ^{32}P -labelled probe 7. In (a) to (c), 1 μg of denatured genomic DNA was applied to a nitrocellulose membrane, and in (d), bacterial colonies scraped from an agar plate were applied to a Biodyne A membrane and hybridized and washed at 40 °C. (a) For the location of the organisms see legend to Fig. 3. (b) Rows A–F, and G1 and G2, *H. ducreyi* strains; G3, *Haemophilus influenzae*; G4, *Escherichia coli*; no DNA was spotted on G5. (c) Rows A–C, and D1–D4, *H. ducreyi* strains; D5, *Haemophilus influenzae*; D6, *Actinobacillus actinomycetemcomitans*. (d) Rows A–C, and D1 and D2, *Haemophilus ducreyi* strains; D3, *Escherichia coli*; no material was applied on D4, D5 and D6.

Table 3. Summary of specificity and reliability tests with *H. ducreyi*-specific DNA probes

Probe	rRNA	Length (bases)	No. positive strains/ no. strains tested		Hybridization and wash temp. (°C)
			<i>H. ducreyi</i>	non- <i>H. ducreyi</i>	
1	16S	25	70/70	0/14	45
2	16S	28	85/85	0/14	50
3	16S	28	89/89	0/14	57
4	16S	29	51/51	0/13	50
5	16S	24	41/41	0/13	55
6	23S	28	68/68	0/14	45
7	23S	20	70/70	0/13	40
8	23S	28	87/87	0/14	40

membranes was used as target as illustrated in Fig. 4 for probe 7. The results are also summarized in Table 3; all probes gave unequivocal positive hybridization signals with all *H. ducreyi* strains tested at the hybridization and wash temperatures indicated. However, as shown in Fig. 3, probe 8 no longer proved reliable when used at higher stringencies.

Discussion

Chancroid and its aetiological agent, *Haemophilus ducreyi*, have gained more interest in the last few years because of its increased incidence in industrialized countries and its association with human immunodeficiency virus infection (Albritton, 1989; Morse, 1989). Generally the diagnosis of chancroid is established on a clinical basis. However, this has proven to be less than adequate since ulcers may also be caused by other infectious agents such as herpes simplex virus or *Treponema pallidum*. Diagnosis based on microscopy of ulcer material after Gram staining or by an indirect fluorescent antibody technique (Denys *et al.*, 1978) is not recommended. Recently developed immunoassays are not entirely satisfactory for a reliable diagnosis of *H. ducreyi* (Museyi *et al.*, 1988; Schalla *et al.*, 1986). The definite diagnosis of *H. ducreyi* can be made only by culture, but the sensitivity of this method is poor. Thus, there is a definite need for a more reliable and rapid diagnostic technique for *H. ducreyi*. DNA-probe technology may fulfil this need. For reasons already mentioned, techniques using probes which target the rRNA are most promising.

In order to develop *H. ducreyi*-specific nucleic acid probes targeting rRNA, part of a rRNA cistron of the type strain (CIP 452^T) was amplified *in vitro* using PCR with primers complementary to conserved regions in the 16S and 23S rRNA genes. Using such primers and a relatively small amount (1 µg) of whole-cell DNA, a 2400 bp fragment could readily be amplified. Unpublished results from our laboratory indicated that large amounts of amplified fragments, up to about 4500 bp in length, can be obtained from a variety of organisms starting from the amount of unpurified DNA present in one bacterial colony. Consequently this technique is particularly useful for obtaining sequence information from micro-organisms such as *H. ducreyi* for which mass cultures are difficult to produce or when the isolation of intact 16S or 23S rRNA molecules is problematic. Sequence comparison of a rRNA gene of the *Neisseria gonorrhoeae* type strain cloned conventionally (Rossau *et al.*, 1988) or cloned via the PCR technique revealed no differences (R. Rossau and others, unpublished observations), thereby confirming the fidelity of the technique as claimed by Medlin *et al.* (1988).

After the amplification step, the fragment was gel-purified, cloned in a plasmid vector, and sequenced as shown in Fig. 1. This approach was favoured over direct sequencing of purified rRNA (Lane *et al.*, 1985) or enzymically amplified material without cloning (Edwards *et al.*, 1989) for several practical reasons. Firstly, mass culture and rRNA purification is avoided since the procedure only requires a small amount of genomic DNA, which can easily be obtained. Secondly, the sequence information coming from purified plasmid templates is very reliable, in contrast to directly sequenced PCR material. The opposite strand is also available for sequencing, in contrast to direct rRNA sequencing. Thirdly, the sequence information obtained is more complete as compared with direct rRNA sequencing; only about 40 nucleotides at the 5' end of the 16S rRNA gene cannot be sequenced. Furthermore, if desired, 23S rRNA sequences and spacer region sequences can also be determined using the same clones. Comparable strategies for sequencing rRNA genes have been successfully employed recently by Medlin *et al.* (1988) and Huss & Sogin (1989). Arguments corroborating the correctness of the rRNA gene sequences shown in Fig. 2 are: (i) conserved nucleotides matched with those of other published sequences (Dams *et al.*, 1988); (ii) a comparable secondary structure could be obtained (see Results); (iii) mutations in stem structures were compensated by other mutations; and (iv) no differences were found in regions sequenced on opposite strands.

Comparison of rRNA molecular sequences, in particular the 16S rRNA, is a very important tool for studying the evolutionary relationships of organisms. Extensive studies have always been hampered by the time-consuming cloning of the rRNA genes. The PCR-assisted cloning of rRNA genes as described in this study and recently by other investigators (Medlin *et al.*, 1988; Huss & Sogin, 1989) offers a fast and reliable alternative to the direct rRNA sequencing method of Lane *et al.* (1985).

In *Bergey's Manual of Systematic Bacteriology*, *H. ducreyi* is listed as an unequivocal *Haemophilus* species in the family Pasteurellaceae (Kilian & Biberstein, 1984). However, its inclusion in the genus *Haemophilus* was solely based on morphological characteristics, X-factor requirements, and the mol% G + C of the genome. By themselves, these features have limited taxonomic significance, and until recently, no substantial evidence was available in support of the present classification of *H. ducreyi*. By contrast, Carlone *et al.* (1988) showed that the isoprenoid quinone structure of *H. ducreyi* is essentially different from that of other *Haemophilus* species. Casin *et al.* (1985) and Albritton (1989) could not detect significant levels of DNA homology between *H. ducreyi* and *Haemophilus*, *Actinobacillus*, or *Pasteurella*

species, nor could Albritton *et al.* (1986) demonstrate significant competition for homospecific transformation with *Haemophilus influenzae*. DNA:rRNA hybridization results recently published by De Ley *et al.* (1990) corroborated these findings and established that *H. ducreyi* was a member of the *Pasteurellaceae* but did not belong to one of the three authentic genera in this family. From the analysis of the rRNA sequences of the type strain of *H. ducreyi* (CIP 542^T), it can be inferred that *H. ducreyi* is related to the *Pasteurellaceae* branch of the gamma subdivision of the *Proteobacteria*. However, *H. ducreyi* seems not to be specifically related to any of the *Actinobacillus*, *Haemophilus*, or *Pasteurella* species used by Chuba *et al.* (1988) (see Table 2); in fact, the homology values obtained with these species suggest a relationship at or below the genus level. This is in complete agreement with the findings of De Ley *et al.* (1990). Nevertheless, the exact taxonomic position of *H. ducreyi* within the *Pasteurellaceae* on the basis of rRNA sequences must await more complete sequence data on more taxa of this complex bacterial family.

A valuable DNA probe for the laboratory diagnosis of chancroid should not hybridize to strains not belonging to the species *H. ducreyi*, and should hybridize to all *H. ducreyi* strains regardless of origin. Lack of reliability is one of the main problems encountered with serodiagnosis of *H. ducreyi*; indeed, Asian material does not cross-react adequately with sera of African origin and vice versa (Museyi *et al.*, 1988). The hybridization results summarized in Table 3 show that all probes tested were highly reliable and completely specific. Except for probe 8, the hybridization experiments were performed at high stringencies (about 5 °C below T_d), but most of the probes did not lose their specificity at lower stringencies (about 10–15 °C below T_d). The fact that all the probes are completely specific suggests a rather distant relatedness of *H. ducreyi* to other taxa tested including six strains of different species in the *Pasteurellaceae*. The high reliability found for all probes tested indicates that *H. ducreyi* is a genotypically homogeneous taxon. This corroborates the DNA:DNA hybridization results of Casin *et al.* (1985). No or only minor sequence differences seem to exist in the rRNA molecules of isolates from different origins, since the probes used were derived from the most variable regions within the rRNA molecules. A higher degree of sequence variability was observed among strains of *H. ducreyi* solely in the 23S rRNA region targeted by probe 8, but it remains to be determined whether this variability is epidemiologically relevant.

The *H. ducreyi*-specific probes described in this study can be used directly on bacterial cells immobilized and briefly lysed on nylon membranes. As such, the probes have applications for culture confirmation of *H. ducreyi*.

However, the probes may be of greater value in the rapid detection and control of chancroid if they can be combined with a non-radioactive detection system enabling noncultural diagnosis in clinical material. Since the *H. ducreyi* probes do not cross-hybridize with relatively closely related species of the genera *Haemophilus*, *Actinobacillus*, or *Pasteurella*, it is highly unlikely that these probes would cross-react with more remotely related organisms which may be found in urogenital samples.

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