BACTERIAL TAXONOMY

Phylogenetic analysis of *Calymmatobacterium granulomatis* based on 16S rRNA gene sequences

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Calymmatobacterium granulomatis is the aetiological agent of granuloma inguinale – a chronic granulomatous genital infection – and is morphologically similar to members of the genus *Klebsiella*. This study determined the 16S rRNA gene sequence of *C. granulomatis* and the taxonomic position of the organism in relation to the genus *Klebsiella*. Genomic DNA was extracted from *C. granulomatis*-infected monocytes and from frozen and formalin-fixed paraffin wax-embedded tissue biopsy specimens from patients with histologically proven granuloma inguinale. The 16S rDNA was amplified by PCR with broad range oligonucleotide primers. The amplified DNA fragments were cloned into *p*MOS vector, digested with *Bam*HI and *Pst*I restriction endonucleases, hybridised with a gram-negative bacterial probe (DL04), sequenced in both directions by the automated ALFTM DNA sequencer, verified on an ABI Prism 377 automated sequencer and analysed with DNASIS and MEGA software packages. Sequence analysis revealed DNA homology of 99% in *C. granulomatis* from the different sources, supporting the belief that the bacteria in the culture and the biopsy specimens belonged to the same species, although there was some diversity within the species. Phylogenetically, the strains were closely related to the genera *Klebsiella* and *Enterobacter* with similarities of 95% and 94% respectively. *C. granulomatis* is a unique species, distinct from other related organisms belonging to the γ subclass of Proteobacteria.

Introduction

Calymmatobacterium granulomatis is a fastidious gram-negative capsulate bacillus found intracytoplasmically within macrophages. It is the aetiological agent of granuloma inguinale, a sexually transmitted disease. The diagnosis of granuloma inguinale is based on the visualisation of 'Donovan bodies' in tissue smears [1]. *C. granulomatis* cannot be cultured on conventional cell-free media, although occasionally successful growth in embryonated eggs [2, 3], and more recently in a monocyte co-culture system, has been achieved [4]. On the basis of ultrastructural morphology and serological characteristics, the organism is considered to be related to members of the genus *Klebsiella* [5–8].

Analysis of 16S rRNA gene sequences is a useful method for the determination of phylogenetic relation-

ships between bacteria and provides a logical basis for taxonomic classification [9–13]. The use of PCR with 16S rRNA primer sequences allows the amplification of bacterial DNA directly from infected host tissue and this DNA can be used for sequence analysis [13–15]. The aims of this study were to determine the 16S rRNA sequence of *C. granulomatis* obtained from monocyte co-culture and infected tissue and to establish the taxonomic position of *C. granulomatis* in relation to the genus *Klebsiella*.

Materials and methods

Clinical specimens

Tissue biopsy specimens from three patients with histologically proven granuloma inguinale were used. One biopsy specimen was co-cultured in monocytes as described previously [4], the second was kept at –70°C until analysis, whilst the third specimen was formalin-fixed and embedded in paraffin wax. A breast tumour biopsy specimen, uninfected monocytes, pyrogen-free water and laboratory strains of *Klebsiella pneumoniae*, *K. oxytoca*, *Serratia marcescens*, *Escherichia coli*,

Received 6 Oct. 1998; revised version accepted 10 Dec. 1998.

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Acinetobacter anitratus and *Staphylococcus aureus* were used as controls.

Extraction of DNA

Frozen tissue and monocyte co-culture. The methods used were modified from those described previously [16]. Fragments of the frozen tissue (*c.* 3 × 5 mm) were rinsed in sterile water, homogenised and placed in 400 µl of digestion buffer (500 mM Tris, pH 9.0, 20 mM EDTA, 10 mM NaCl and SDS 1%) with proteinase K (Boehringer Mannheim, Germany) at a final concentration of 1 mg/ml; 500 µl of the monocyte co-culture suspension were treated similarly. The samples were incubated at 56°C for 18 h with agitation. The proteinase K was then heat inactivated at 95°C for 10 min and ribonuclease A (Boehringer Mannheim) was added to a final concentration of 4 µg/ml. The samples were incubated at room temperature for 30 min and then extracted three times with equal volumes of Tris-buffered phenol:chloroform:isoamylalcohol (25:24:1). The DNA was precipitated with absolute ethanol, washed with ethanol 70%, air dried and resuspended in 50 µl of water.

Formalin-fixed paraffin wax-embedded tissue. The method used was that described by Wright and Manos [17]. Sections (10 µm) from the paraffin wax-embedded tissue were treated with octane and ethanol and then placed in 400 µl of digestion buffer with proteinase K. DNA was extracted and precipitated after digestion and heat inactivation was carried out as for frozen biopsy specimens.

DNA amplification and detection

PCR primers. Broad range oligonucleotide primers directed to positions 8–1390 of the *E. coli* 16S rRNA gene [18] were synthesised by the solid phase phosphoramidite method with a Gene Assembler DNA synthesiser (Pharmacia LKB Biotechnology, USA). Forward primer 8F (5'-AGTTTGATCCTGGCTCAG-3') and reverse primer 13R (5'-AGGCCCGGGAACG-TATTCAC-3') resulted in the amplification of a fragment of 1382 bp.

PCR amplification. PCR amplification of DNA from frozen tissue, paraffin wax-embedded tissue and monocyte co-culture was performed in a volume of 100 µl containing 10 µl of 10 × PCR buffer (100 mM Tris-HCl, pH 8.5, 20 mM MgCl₂, 500 mM KCl), 10 mM of each dNTP; 1.0 U of *Taq* polymerase (Boehringer Mannheim); 20 pmoles of each primer and 5 µl of the DNA template. Amplification was performed in a Gene AmpTM PCR System 9600 (Perkin Elmer, Norwalk, CT, USA) with the following protocol: incubation at 95°C for 5 min and thereafter 40 cycles of 95°C for 1 min (denaturation); 55°C for 1 min (annealing); followed by 72°C for 2 min (extension). The amplicons were kept at -20°C until further analysis. The

amplified DNA fragments were separated by electrophoresis through an agarose 1.5% gel containing ethidium bromide and visualised under UV light. The amplified products were excised and eluted from the gel with a Sephaglass BandPrep kit (Pharmacia LKB Biotechnology) and cloned by the T-cloning method with a pMOSblue T-vector kit (Amersham Life Science, USA) according to the manufacturer's instructions.

Hybridisation experiments

The oligonucleotide probes used were the universal bacterial probe RDR245# 5'-GTACAAGGCCCGG-GAACGTATTCACCG-3' 1369–1395, the gram-negative probe DLO4# 5'-GACGTAAGGGCCATGATGAC-TTGACGTC-3' 1190–1217 and the gram-positive universal probe RWO3# 5'-GACGTCAAATCATCAG-CCCCTTATGTC-3' 1190–1217 as described by Greisen *et al.* [19]. The probes were labelled by the digoxigenin (DIG) 3'-labelling method (Boehringer Mannheim). After electrophoresis, the gels were immersed in denaturation solution (0.5 N NaOH, 1.5 M NaCl) twice for 15 min, rinsed briefly in distilled water, immersed in neutralisation buffer (0.5 M Tris-HCl, 3 M NaCl) twice for 15 min at room temperature. The DNA fragments were transferred by vacuum to a Hybond N+ nylon membrane (Amersham, Life Science) which had been soaked in sterile distilled water. After transfer, the DNA was cross-linked to the membrane for 3 min under UV light at 254 nm. The membranes were pre-hybridised with blocking reagent 1%, 100 mM maleic acid, 150 mM NaCl at 68°C for 1 h. The DIG-labelled probe (10 pmol/ml) was prepared in 5 × sodium chloride sodium citrate (SSC), blocking reagent 1%, N-lauroylsarcosine 0.1% w/v, SDS 0.02% w/v and placed in hybridisation bags with the membrane, and hybridised for 3 h at 68°C with constant mixing. At the end of hybridisation, the solution was removed from the bag and the membranes were washed twice for 5 min in 2 × SSC, SDS 0.1% at room temperature to remove any unbound probe. Thereafter the membranes were washed twice for 15 min, in 0.1 × SSC, SDS 0.1% at 68°C.

The immobilised DNA fragments on the nylon membranes were detected with the Nucleic Acid Detection kit (Boehringer Mannheim) with nitroblue tetrazolium (NBT) and X-Phosphate (BCIP) (50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt in dimethylformamide). The membranes were washed briefly for 1 min in 100 mM maleic acid, 150 mM NaCl and Tween 20 0.3% w/v, and then incubated for 30 min in blocking reagent 1%, 100 mM maleic acid, 150 mM NaCl. The membranes were incubated for 30 min with anti-digoxigenin-alkaline phosphatase conjugate (150 mU/ml). Unbound antibody conjugate was removed by washing twice for 15 min with 100 ml of buffer. The membranes were equilibrated for 2 min with 100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl₂, pH 9.5, and

incubated with freshly prepared colour substrate (200 μ l NBT/BCIP stock solution in 100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl₂, pH 9.5) in a sealed plastic bag in the dark without shaking. The reaction was stopped by washing the membranes with 50 ml of 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, after sufficient colour development was observed in the DNA fragments.

Sequence determination

All ampicillin-resistant transformants were screened for the appropriate recombinant plasmids by the standard alkaline lysis method [20] and plasmid DNA was prepared with the Qiagen Plasmid Maxi kit (Qiagen, USA). The DNA from multiple clones was purified and concentrated through a Microcon 100 microconcentrator (Amicon, USA). The double-stranded DNA templates were sequenced in both orientations with fluorescently labelled M13 and T7 sequencing primers. Sequencing was performed with an automated ALFTM DNA sequencer (Pharmacia LKB Biotechnology) and the Cy5TM Autoread sequencing kit (Pharmacia LKB Biotechnology). All sequences were repeated and verified on an ABI Prism 377 Automated sequencer by Genomix (USA).

Sequence analysis

The three sequences were compared and aligned to known 16S rDNA sequences in the GenBank database with DNASIS v2.1 (Hitachi Software, CA, USA) [21] and MEGA (Molecular Evolutionary Genetics Analysis) [22].

Results

A 1382-bp fragment was observed in each of the samples from frozen tissue, paraffin wax-embedded tissue biopsy specimen and monocyte co-culture with primers 8F and 13R. Hybridisation of the 1382-bp fragment obtained from *Bam*HI and *Pst*I digestion of the recombinant clones with the universal bacterial probe (RDR245) showed signals with all three samples as well as with DNA from both gram-negative and gram-positive bacteria. The gram-negative probe (DLO4) hybridised to the DNA from gram-negative organisms as well as the three samples, but not to gram-positive organisms (Fig. 1a). The universal gram-positive bacterial probe (RWO3) hybridised to gram-positive organisms only and not to the DNA from the three samples or from gram-negative organisms (Fig. 1b).

Fig. 1. Agarose 1% gel electrophoresis of restricted recombinant clones and amplified products obtained from bacterial DNA, hybridised with (a) gram-negative bacterial probe DLO4; (b) gram-positive bacterial probe RWO3. Lane 1, frozen biopsy specimen (KH 6); 2, monocyte co-culture (KH 22); 3, formalin-fixed, paraffin wax-embedded biopsy specimen (KH 34); 4, *E. coli*; 5, *K. pneumoniae*; 6, negative PCR control; 7, *K. oxytoca*; 8, *S. marcescens*; 9, *A. anitratus*; 10, *S. aureus*; 11, *S. aureus*; 12, DIG-labelled DNA mol.wt marker VI (pBR 328 DNA-*Bgl*I + pBR 328 DNA-*Hin*II).

Sequence analysis of 1382-bp fragments of the three samples (~90% of the 16S rRNA gene) revealed 99% homology. Single nucleotide differences observed between the sequences of the three samples were found at varying positions, as indicated in Table 1.

Alignment of the 16S rDNA sequences of *C. granulomatis* was made with sequences of the members of the Enterobacteriaceae and of representatives of several groups of bacteria of the α , β and γ subgroups of Proteobacteria (GenBank). Similarities of 95% and 94% were observed with *K. pneumoniae* and *Enterobacter* spp., whereas only 69% and 58% similarities were observed for *Neisseria gonorrhoeae* and *Bacillus subtilis* strains, respectively. The results of the comparison with other representative bacteria of the α , β and γ subgroups of Proteobacteria are shown in Table 2.

Table 1. 16S rDNA sequence differences derived from bacteria from the monocyte co-culture specimen (KH 22), frozen (KH 6) and formalin-fixed, paraffin wax-embedded (KH 34) tissue biopsy specimen

Nucleotide no.	Bacterial strain		
	KH 22	KH 6	KH 34
171	C	T	T
445	A	T	T
609	C	T	T
837	T	C	C
941	T	G	T
943	G	T	◆
966	◆	C	C
968	T	T	A
983	C	C	A
1005	T	T	◆
1919	A	A	◆
1034	A	A	C
1065	A	A	◆
1097	C	◆	C
1141	T	T	C
1288	A	G	G

◆, indicates deletion.

Table 2. Levels of 16S rDNA sequence similarities between *C. granulomatis* and some references species belonging to the α , β and γ subclasses of Proteobacteria

Bacterial species with GenBank accession nos.	Sequence similarity to <i>C. granulomatis</i> (%)*
<i>Escherichia coli</i> A14565	91
<i>Klebsiella pneumoniae</i> X87276	95
<i>Serratia marcescens</i> M59160	91
<i>Enterobacter</i> sp. U39556	94
<i>Citrobacter freundii</i> M59291	91
<i>Salmonella typhimurium</i> U90316	91
<i>Hafnia alvei</i> M59155	91
<i>Erwinia amylovora</i> X83265	91
<i>Erwinia carotovora</i> M59149	91
<i>Yersinia enterocolitica</i> Z49829	90
<i>Yersinia aldovae</i> X75277	87
<i>Acyrtosiphon pisum</i> symbionts M27040	90
<i>Rhizobium leguminosarum</i> X67233	60
<i>Neisseria gonorrhoeae</i> X07714	69
<i>Caulobacter crescentus</i> M83799	58
<i>Bacillus subtilis</i> X60646	58

*Values are based on the results of 1382 nucleotides.

The phylogenetic methods placed *Klebsiella* as the most closely related genus with strong bootstrap values. Fig. 2 shows the phylogenetic relationships among various taxa, as determined by the neighbour-joining method. All phylogenetic trees formed a cluster of the *C. granulomatis* sequences and this cluster was distinct from *Klebsiella* and other members of the Enterobacteriaceae. The relationship of this group to *Klebsiella* shows the evidence for the new genus *Calymmatobacterium* being within the taxon Enterobacteriaceae. On the basis of the phylogenetic findings, *C. granulomatis* is a member of a new genus, for which the name *Calymmatobacterium* is appropriate.

The sequence signatures as suggested by Woese [23] for the α , β and γ subclasses of Proteobacteria form distinct groups. Those of the three 16S rDNA sequences of *C. granulomatis* clustered within the γ subclass. The 16S rRNA sequences determined for *C. granulomatis* have been deposited in the Genome Sequence Database (GSDP) at Los Alamos National Laboratory (formerly GenBank) under the following accession numbers: *C. granulomatis* monocyte co-culture (strain KH 22), AF010251; *C. granulomatis* frozen biopsy specimen (strain KH 6), AF010252; *C. granulomatis* from formalin-fixed paraffin wax-embedded biopsy specimen (strain KH 34), AF010253.

Discussion

The laboratory diagnosis of granuloma inguinale is difficult. It relies only on the observation of 'Donovan bodies' in clinical specimens, which has a sensitivity of <70% [1]. The absence of any serological identification tests and the inability to cultivate *C. granulomatis* *in vitro* has hampered the identification and characterisation of this organism.

In-vitro amplification technology has become a powerful tool for the rapid analysis of specific areas of DNA or even entire genes. This is the first report in which the 16S rDNA of *C. granulomatis* – a previously uncultured organism implicated in the aetiology of granuloma inguinale – was amplified, cloned and sequenced from monocyte co-cultures, frozen and formalin-fixed paraffin wax-embedded tissue biopsy specimens.

Assays based on nucleic acid detection have the potential for greater sensitivity than immunological methods. In this study, oligonucleotide probes designed for the detection and identification of bacterial pathogens in normally sterile body fluids were used in the hybridisation of the restricted recombinant clones from the specimens. Hybridisation experiments performed with the specific universal bacterial (RDR245) and gram-negative (DLO4) oligonucleotide probes suggested that the restriction endonuclease-digested fragments from the recombinant clones from all three

Fig. 2. Phylogenetic relationship of *C. granulomatis* (KH 22, KH 6 and KH 34) to other bacteria of the α , β and γ subgroups of Proteobacteria. The tree was constructed by the neighbour-joining method using MEGA. The root of the tree was determined by using the sequence of *B. subtilis* as an outgroup. The tree is based on a comparison of 1382 bp.

sources originated from eubacteria. Because no hybridisation signal was obtained with the gram-positive probe (RWO3), it was concluded that the organisms were gram-negative and not gram-positive. The gram-positive probe (RWO3) corresponds to two sequence signatures identified in the 16S rRNA [19], one being a C residue at position 1207 and the other an A residue at position 1198 of the *E. coli* 16S rRNA gene. The residues of the organisms at these positions were G and T respectively. This provides further confirmation that the sequence was not from a gram-positive organism.

The comparative analyses of nucleic acid sequences coding for the 16S rRNA gene are useful for the study of phylogenetic relationships among many organisms which reflect evolutionary pathways and for characterisation of organisms of uncertain affiliation [24]. For

any meaningful analysis, a part of at least 1000 bp of the 16S rRNA gene needs to be sequenced [25]. In this study, 1382 bp of the 16S rDNA (~90% of the total primary rRNA structure) were sequenced, thus permitting the sequence analysis to be undertaken with some degree of confidence.

Little is known about intra-species variations of 16S rDNA sequences. Such diversity of sequences for one species may be caused by species heterogeneity, heterogeneity of the 16S rRNA copies [14] within a strain, or sequencing errors. The 16S rDNA sequences of *C. granulomatis* appeared to be relatively stable, but as only a few samples were studied, no firm conclusion could be made. There are a number of possibilities for the observed sequence differences. There is evidence of very little diversity of the 16S rDNA sequences of

Francisella [26] and *Legionella pneumophila* [27], whereas for *Borrelia burgdorferi* strains, up to 1.0% divergence has been demonstrated [28]. Eckloff *et al.* [29] compared the 16S rDNA sequences from five isolates of *Helicobacter pylori* and found deviations from the consensus sequence at 13 separate positions which could be attributed to clonal origins. Relman *et al.* [14] reported the unique 16S rRNA gene sequences from tissues of three of four unrelated patients with bacillary angiomatosis, whilst a sequence obtained from the tissue of the fourth patient differed from that of the other three patients at four of 241 base positions. It is suggested that these sequences are all related to the 16S rRNA sequence of the same organism, but the sequence heterogeneity might indicate the presence of related strains within diseased tissue. Similarly, a small number of nucleotide differences are known to exist within the several copies of the *E. coli* rRNA gene. Another explanation for sequence difference is the fidelity of *Taq* polymerase. *Taq* polymerase could misincorporate nucleotides, particularly during the amplification of damaged DNA, which would be observed after direct sequencing of PCR products. This emphasises the importance of cloning and sequence analyses of multiple clones of the amplified products from the same and different specimens in order to obtain a 'near-perfect' sequence [12]. It would be interesting to consider whether the few differences observed in the 16S rDNA sequences of *C. granulomatis* strains are reflected in phenotypic traits.

In this study, sequence analysis of the 16S rDNA was able to identify bacteria from all three sources accurately as belonging to the γ subclass of Proteobacteria, the genus being unique. Comparative sequence analysis is easy to interpret when a newly characterised sequence can be compared with a known sequence available in the database. However, at present, none of the obtained sequences could be compared with any known sequences of *C. granulomatis* from the available databases as these were unavailable. The phylogenetic tree analysis confirmed that *C. granulomatis* belonged to a separate branch of the γ subclass of the Proteobacteria which is characterised by species belonging to the genera [23].

Although the causative agent of granuloma inguinale has been proposed to be morphologically related to *Klebsiella* [8], and the organisms are phylogenetically related to some extent, *Klebsiella* isolates were clearly distinct. *Klebsiellae* can be cultivated readily on cell-free media whereas calymmatobacteria are fastidious organisms and do not grow on cell-free medium. The phenotypic characteristics of *klebsiellae* do not resemble those of calymmatobacterium. Currently, there are no phenotypic characteristics that could easily and readily be used by clinical laboratories to differentiate calymmatobacteria from other members of the Enterobacteriaceae, particularly *klebsiellae*. In addition, there are no conventional or commercial tests that

can identify and differentiate these organisms reliably. The use of PCR with specific primers targeting the 16S rRNA gene should accurately identify and differentiate strains of *C. granulomatis* from other members of the Enterobacteriaceae. This method would provide a valuable and efficient technique which could be used by clinical microbiologists. Alternatively, the specific sequences could be used for the development of a probe for use in DNA immunoassays.

Forsman *et al.* [26] observed unusually high levels of sequence homology between the 16S rRNA sequences of *Wolbachia persica* and *Francisella* and suggested that instead of placing *W. persica* within the genus *Francisella*, the species should be further characterised phenotypically to determine its relationship to *Francisella* species. In a similar way, although the 16S rDNA sequences of *C. granulomatis* are closely related to those of *Klebsiella*, they form a distinct group within the taxon Enterobacteriaceae. Thus it would be appropriate to retain the genus *Calymmatobacterium*. The differential phenotypic characteristics of the two groups support this proposal.

This work was supported by grants from the University of Natal research fund and the Medical Research Council of South Africa.

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