BACTERIAL TAXONOMY

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

A. B. M. KHARSANY, A. A. HOONSEN, P. KIEPIELA, R. KIRBY* and A. W. STURM

MRC Genital Ulcer Disease Research Unit, Department of Medical Microbiology, Faculty of Medicine, University of Natal, Kwa Zulu Natal and *Department of Biochemistry and Microbiology, Rhodes University, Grahamstown, South Africa

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 pMOS vector, digested with BamHI and PstI restriction endonucleases, hybridised with a gram-negative bacterial probe (DL04), sequenced in both directions by the automated ALF™ 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 aetiologic 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 relationships 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,
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:isoamyl-alcohol (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’-AGTTTGATCCTGGCTCA-3’) and reverse primer 13R (5’-AGGCCCGGGAACGTAATTCACCG-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 pmol of each primer and 5 μl of the DNA template. Amplification was performed in a Gene Amp™ PCR System 9600 (Perkin Elmer, Norwalk, CT, USA) with the following program: 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’-GTACAAGGCCCCGGGAACGTATTCCACCG-3’ 1369–1395, the gram-negative probe DLO4# 5’-GAGCTTAAAGGCCATGATGAC-TTGACGTC-3’ 1190–1217 and the gram-positive universal probe RW03# 5’-GACGCAATACGATC-CCCTTTATGTC-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 μU/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 µl 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 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 BamHI and PstI 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 (DL04) 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 (WO3) hybridised to gram-positive organisms only and not to the DNA from the three samples or from gram-negative organisms (Fig. 1b).

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 ALF DNA sequencer (Pharmacia LKB Biotechnology) and the Cy5 Autoread sequencing kit (Pharmacia LKB Biotechnology). All sequences were repeated and verified on an ABI Prism 377 Automated sequencer by Genomis (USA).
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 monoculture co-culture specimen (KH 22), frozen (KH 6) and formalin-fixed, paraffin wax-embedded (KH 34) tissue biopsy specimen

<table>
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<th>Bacterial strain</th>
<th>KH 22</th>
<th>KH 6</th>
<th>KH 34</th>
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</table>

*, indicates deletion.

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

*Values are based on the results of 1382 nucleotides.
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 bacillary angiomatosis, whilst a sequence obtained which could be attributed to clonal origins. Relman et al. [28] observed 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.

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References

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