Phylogenetic evidence for reclassification of *Calymmatobacterium granulomatis* as *Klebsiella granulomatis* comb. nov.

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By sequencing a total of 2089 bp of the 16S rRNA and *phoE* genes it was demonstrated that *Calymmatobacterium granulomatis* (the causative organism of donovanosis) shows a high level of identity with *Klebsiella* species pathogenic to humans (*Klebsiella pneumoniae*, *Klebsiella rhinoscleromatis*). It is proposed that *C. granulomatis* should be reclassified as *Klebsiella granulomatis* comb. nov. An emended description of the genus *Klebsiella* is given.

**Keywords:** *Calymmatobacterium*, *Klebsiella*, sequence data, phylogenetic inferences

*Calymmatobacterium granulomatis* is the presumed causative agent of donovanosis, an important cause of genital ulceration that occurs in small endemic foci in all continents except Europe and Antarctica. The name *C. granulomatis* was originally given to the pleomorphic bacterium cultured from donovanosis lesions by Aragão & Vianna (1913). Although these early cultures are highly dubious and were probably not of the organism itself (Richens, 1985), the name *C. granulomatis* has retained precedence over others.

*C. granulomatis* is known to be an encapsulated, non-motile, facultatively anaerobic, Gram-negative bacterium (Chandra & Jain, 1991; Davis, 1970; Davis & Collins, 1969; Dodson et al., 1974; Kuberski et al., 1980). *C. granulomatis* has been associated with the genus *Klebsiella* because of the above characteristics and common antigenicity (Maddocks et al., 1975; Packer & Goldberg, 1950). Furthermore, two members of the genus *Klebsiella* that produce clinical disease and pathologic changes very similar to those of *C. granulomatis* are *Klebsiella rhinoscleromatis* (Levine & Hoyt, 1947; Shaw & Martin, 1961; Welsh et al., 1963), which is implicated in a granulomatous disease of the nose, and *Klebsiella ozaenae* (Richens, 1985) which is implicated in chronic atrophic rhinitis. Richens (1985) goes so far as to place *C. granulomatis* in the genus *Klebsiella*, although difficulties in cultivating the organism (Richens, 1991) have prevented further characterization of this relationship.

Non-cultivable pathogenic eubacteria have been identified by PCR using primers targeting conserved genes (Fredricks & Relman, 1996). We have shown through sequencing a 334 bp region of the phosphate porin (*phoE*) gene that *C. granulomatis* has a high degree of molecular identity with other *Klebsiella* species in this region (Bastian & Bowden, 1996). We present here an analysis of the almost complete 16S rRNA and *phoE* sequences for *C. granulomatis*, *K. rhinoscleromatis* and *Klebsiella pneumoniae*.

At least two punch biopsies or swabs were obtained from the lesions of eight patients with clinical donovanosis. One punch biopsy was fixed and examined for Donovan bodies by the slow Giemsa technique (Sehgal & Jain, 1987) whilst DNA was extracted from the second punch biopsy (seven samples) (Mullenbach et al., 1989) or swab (one sample) (Beige et al., 1995). DNA was also obtained from type cultures of *K. pneumoniae* [NCTC 9633?] (National Collection of Type Cultures) and *K. rhinoscleromatis* (NCTC 5046?).

Primer sets used in this study include (5’-ACCTACGCAACACCGACTCTCTCGG-3’ and 5’-TGA-TCAGAAGTGATTGGTCAT-3’), 604 bp *phoE* prod-
**Table 1.** Strain designations and accession numbers for sequences obtained from the GenBank and EMBL databases, and for sequences novel to this study

Abbreviations: NCTC, National Collection of Type Cultures; ATCC, American Type Culture Collection; DSM, DSMZ – Deutsche Sammlung für Mikroorganismen und Zellkulturen; JCM, Japanese Collection of Microorganisms; PAH, Princess Alexandra Hospital, Brisbane, Australia; \(^*\), type Culture.

<table>
<thead>
<tr>
<th>Organism</th>
<th>16S rRNA</th>
<th>phoE</th>
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<tr>
<td></td>
<td>Strain</td>
<td>Accession</td>
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<tr>
<td><em>Calymmatobacterium granulomatis</em></td>
<td>*</td>
<td>AF009171(^*)</td>
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<tr>
<td><em>Klebsiella rhinoscleromatis</em></td>
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<td>AF009169(^*)</td>
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<td>DSM 30104(^*)</td>
<td>X87276</td>
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<tr>
<td><em>Klebsiella ornithinolytica</em></td>
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<td>AB004756</td>
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<td>X93215</td>
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<td>PAH11472</td>
<td>X68674</td>
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* Sequences obtained from clinical specimens.
\(^*\) Sequences novel to this study.
\(^\ddagger\) Strain not specified in GenBank report.

One microlitre of template DNA was included in a 25 \(\mu\)L PCR mixture that comprised the following solution: 10 mM Tris/HCl pH 8.0, 50 mM KCl, 2.5 mM MgCl\(_2\), 0.01% gelatin, 0.2 \(\mu\)M dNTPs, 0.5 \(\mu\)M primers and 0.5 Units Tag polymerase. Samples were subjected to 30 cycles of denaturation (94 \(^\circ\)C, 40 s), annealing (55 \(^\circ\)C, 40 s) for phoE primers; 52 \(^\circ\)C, 40 s for 16S primers) and extension (72 \(^\circ\)C, 60 s) in a thermal cycler (Corbett Research, Sydney). All steps of PCR template preparation, PCR mix and gel analysis were conducted in three separate dedicated workrooms. Template negative controls were included in each reaction. Products of the expected size were purified and sequenced in both directions using either the dye terminator method (Applied Biosystems) or a manual T7 sequencing kit (Pharmacia-Biotech).

Initially, a 604 bp region of phoE was amplified and sequenced from 4/4 patients with histologically confirmed donovanosis and from 4/4 patients with negative histology for whom donovanosis remained the clinical diagnosis. The same *Klebsiella*-like sequence was amplified from all eight patients thereby confirming and extending the results obtained previously (Bastian & Bowden, 1996).

In order to further investigate this relationship we determined the sequence of a coding region spanning 95% of the phoE gene (1001/1059 bp) from *K. pneumoniae*, *K. rhinoscleromatis* and *C. granulomatis* isolates, along with sequences for a 1088 bp region of the 16S rRNA from *C. granulomatis* and *K. rhinoscleromatis*. The 16S rRNA sequence of *K. pneumoniae* [DSM 30104\(^T\) (DSMZ, Deutsche Sammlung für Mikroorganismen und Zellkulturen)] was obtained from the GenBank database. These sequences were aligned with the sequences of other *Klebsiella* sp. and related *Enterobacteriaceae* available from the GenBank/EMBL databases using the program CLUSTAL W (Thompson et al., 1994). Due to the limited amount of complete phoE sequence data available, *Klebsiella planticola*, *Klebsiella ornithinolytica* and *Yersinia enterocolitica* were only included in the 16S rRNA alignment. For similar reasons different species of *Enterobacter* have been compared for the 16S rRNA and phoE genes.

Phylogenetic analyses was performed using the PHYLIP software package (Felsenstein, 1993). Programs employed included SEQBOOT: to produce multiple data sets by bootstrap resampling (100 iterations); DNADIST: to compute distances between species for all data sets with the Jukes–Cantor algorithm; NEIGHBOR: to produce 1000 trees (100 data sets × 10 jumbled input
Phylogenetic analysis of C. granulomatis

Fig. 1. Phylogenetic trees showing the relationship of C. granulomatis, members of the genus Klebsiella and some related members of Enterobacteriaceae. Bootstrap values, expressed as percentages, are given at branch points. Yersinia enterocolitica and Serratia marcescens were used as the outgroups for 16S rRNA and phoE analysis respectively: (a) 16S rRNA, comparison of 1088 bp; (b) phoE, comparison of 1001 bp. All GenBank/EMBL accession numbers and strain designations are shown in Table 1.

orders) with the neighbour-joining method; CONSENSE: to compute the strict consensus tree by the majority-rule consensus tree method.

Accession numbers for sequences obtained from the GenBank/EMBL databases and for sequences novel to this study are provided in Table 1 with the strain designations. Phylogenetic trees, including the confidence values of branching are shown as cladograms in Fig. 1 for the 16S rRNA and phoE. It is evident from tree topologies that C. granulomatis has a close phylogenetic relationship with members of the Klebsiella genus known to be human pathogens (K. rhinoscleromatis, K. pneumoniae). Bootstrap analysis data confirmed that this association was highly significant for both the phoE and the 16S rRNA genes (bootstrap values 100%). Nucleotide similarities between the three isolates ranged from 98.8–99.8% for the 16S rRNA and 99.7–99.8% for phoE.

PCR-based identification of non-cultivable microbial pathogens may be confounded by the incidental amplification of colonizing non-pathogenic bacteria. Fredricks & Relman (1996) have proposed several criteria for establishing microbial disease causation by molecular methods. Their guidelines stipulate that: the sequence-based identification should be reproducible; the nucleic acid sequence should be found at its highest concentration in diseased tissue but be absent, or present at only low copy numbers, in normal tissue; the relationship should be biologically plausible with the known phenotypic characteristics of the non-cultivable organism; and ideally the molecular identification should be established at the cellular level by in situ hybridization.

The studies described in this and our other papers (Bastian & Bowden, 1996; Carter et al., 1997, 1999) fulfil these criteria for associating the causative organism of donovanosis with the genus Klebsiella. In this paper, the clinical samples from all eight patients with clinical donovanosis produced PCR products that demonstrated greater than 99% similarity with K. pneumoniae and K. rhinoscleromatis (i.e. the association is reproducible). We have developed a diagnostic PCR based on the observation that two unique base changes in the phoE gene of C. granulomatis eliminate HaeIII restriction sites (Carter et al., 1999). All 14 clinical donovanosis samples tested with this diagnostic method gave the restriction digest profile expected from sequence data. No products were obtained from patients with unrelated genital conditions (i.e. the molecular identification is specific). As described earlier, this molecular association of C. granulomatis with the Klebsiella genus is biologically plausible based on the known phenotypic and antigenic characteristics of the organism. Finally, though in situ hybridization experiments have not been pursued to demonstrate a tissue–sequence correlation at the cellular level, we have recently been able to cultivate C. granulomatis in a human epithelial cell line. DNA obtained from the cultured organisms had an identical phoE sequence to that obtained from clinical specimens (Carter et al., 1997).

We believe that in light of our data it is reasonable to consider reclassifying C. granulomatis as Klebsiella granulomatis.
Emended description of the genus Klebsiella

Klebsiella species are facultatively anaerobic, Gram-negative, non-motile, generally straight rods arranged singly, in pairs or in short chains and measuring 0.3-1.0 μm in diameter and 0.6-6.0 μm in length (Orskov, 1984). K. granulomatis is pleomorphic (Dienst & Bronwell, 1984), i.e. curved or straight rods, coccoid, diplococcoid, ovoid or elliptical in shape. Most Klebsiella species are capsulated (Orskov, 1984) though K. granulomatis may be capsulated (mature form) or non-capsulated (immature form) (Hart, 1997). Most species are cultivable on routine microbiological media (Orskov, 1984). K. granulomatis is facultatively intracellular, residing within the cytoplasm of large mononuclear cells and cannot be cultured on routine microbiological media (Dienst & Bronwell, 1984). There are currently five recognized species of Klebsiella: K. pneumoniae, Klebsiella oxytoca, Klebsiella terrigena, Klebsiella planticola and Klebsiella ornithinolytica (Orskov, 1984; Sakazaki et al., 1989) and three recognized subspecies of K. pneumoniae: K. pneumoniae subsp. pneumoniae, K. pneumoniae subsp. ozaenae and K. pneumoniae subsp. rhinoscleromatis (Orskov, 1984). In the absence of DNA-DNA hybridization studies it is not possible to determine whether K. granulomatis is a sixth Klebsiella species or a fourth subspecies of K. pneumoniae. For a complete description of the characteristics of the genus Klebsiella see Orskov (1984).

Description of Klebsiella granulomatis comb. nov.

Klebsiella granulomatis (gran.u.lo'ma.tis. L. dim. n. granulum a small grain; Gr. suff. -oma a swelling or tumour; M.L. n. granuloma a granuloma; M.L. gen. n. granulomatis of a granuloma).

Cell characteristics. Gram-negative (Dienst & Bronwell, 1984). Non-sporulating (Richens, 1985). Non-motile (Dienst & Bronwell, 1984). K. granulomatis is pleomorphic when observed in a single plane (Dienst & Bronwell, 1984), i.e. curved or straight rods, coccoid, diplococcoid, ovoid or elliptical in shape. Mature forms are capsulated, ovoid to elliptical in shape and measure 0.5-0.7 μm in diameter and 1.0-1.5 μm in length (Rajam & Rangiah, 1954; Sehgal & Sharma, 1992; Hart, 1997). Immature forms are non-capsulated, coccoid, diplococcoid or bacillarly in shape and measure 0.6-1.0 μm in length (Rajam & Rangiah, 1954; Sehgal & Sharma, 1992; Hart, 1997). Immature, non-capsulated forms may appear as closed safety pins with certain stains due to bipolar chromatin densities (Rajam & Rangiah, 1954; Sehgal & Sharma, 1992; Hart, 1997). Division is via invagination of the cell wall and cytoplasmic membrane (Anderson et al., 1945; Davis & Collins, 1969; Spagnolo et al., 1984; Sehgal & Sharma, 1992). Filamentous processes recognized as pili and fimbriae are present on the surface of most organisms (Dodson et al., 1974; Kuberski et al., 1980; Chandra & Jain, 1991). Numerous round vesicles or blebs endogenous to the cell wall are often seen and may be attached to or detached from the cell wall (Dodson et al., 1974; Kuberski et al., 1980; Chandra & Jain, 1991).


Cultural and growth conditions. K. granulomatis is facultatively intracellular, residing in the cytoplasm of large mononuclear cells and occasionally within polymorphonuclear leukocytes (Dodson et al., 1974; Kuberski et al., 1980; Spagnolo et al., 1984; Chandra & Jain, 1991). In the yolk sac of the developing chick embryo a conspicuous feature is its residence within epithelial cells (Anderson, 1943). Successful cultures have been achieved in vivo in the yolk sac of developing chick embryos (Anderson, 1943) and in the developing chick embryo brain (Thomison, 1951). Cultures in vitro have been achieved utilizing fresh yolk containing embryonic chick heart (Anderson, 1943); chick embryo amniotic fluid (Anderson et al., 1945); semi-solid medium containing peptone, tryptone, dextrose, sea salt, agar and fresh yolk (Dienst, 1948); slants prepared from beef heart infusion agar and fresh yolk (Dunham & Rake, 1948); Locke-Yolk agar with a Locke solution overlay (Dulaney et al., 1948); thioglycollate broth with lactalbumin hydrolysate (enzymic digest of albumin) or Phytone (enzymic digest of soya meal) added (Goldberg, 1959); fresh mononuclear cells (Kharsany et al., 1996; 1997) and a human epithelial cell line (Carter et al., 1997). The optimal temperature for growth of K. granulomatis is 37°C (Anderson, 1943; Beveridge, 1946; Dienst & Bronwell, 1984). Two factors present within the yolk sac of developing chick embryos have been found to be essential for growth. These are a micro-aerophillic environment (Anderson, 1943; Dienst, 1948; Goldberg, 1959) and a polypeptide present in the enzymic digests of bovine albumin and soya meal (Goldberg, 1959).

Storage conditions. K. granulomatis will not remain viable when stored at 5 or 37°C (Anderson, 1943). It has been reported that stock egg yolk cultures remain viable for extended periods when stored at 25°C (Anderson, 1943) though Dienst (1948) could only maintain viability of cultures in fresh yolk medium for 8–10 d at 25°C.

Genetic data. The mol% G+C content is unavailable due to the absence of DNA–DNA hybridization studies. K. granulomatis shows 99.7–99.8% nucleotide similarity with K. pneumoniae and K. rhinoscleromatis, respectively, in the gene encoding the outer-membrane phosphate porin and 98.8–99.8% nucleotide similarity with K. pneumoniae and K. rhinoscleromatis, respectively, in the 16S rRNA gene.

Pathogenicity and habitat. K. granulomatis is not pathogenic for mice, dogs, chickens, the chorioallantoic membrane of chick embryos, rabbits, guinea pigs, Macacus rhesus monkeys, sheep, goats, pigs or...
cows (Anderson, 1943; Anderson et al., 1945; Beveridge, 1946; Dienst et al., 1949). With the exception of the developing chick embryo (and other types of eggs) *K. granulomatis* is pathogenic only for humans (Dienst & Bronwell, 1984) where infection results in the chronic genital ulcerative disease known as donovanosis. *K. granulomatis* has been assumed to be sexually transmitted (Rajam & Rangiah, 1954; Sengupta, 1981; Spence, 1988; Sehgal & Sharma, 1992; Hart, 1997) though an enteric habitat has been postulated (DeMonbreun & Goodpasture, 1933; Dunham & Rake, 1948; Goldberg, 1962, 1964). Goldberg (1962) isolated and cultured *K. granulomatis* from the faeces of a patient with donovanosis and DeMonbreun & Goodpasture (1933) also claim to have achieved successful isolation (though not culture) of *K. granulomatis* from the faeces of 2/4 patients with donovanosis. It is currently unknown whether *K. granulomatis* also has a natural environmental habitat.

**Type culture.** Due to difficulties encountered in the storage of *K. granulomatis*, no type culture is currently available.

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**References**


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