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

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**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.

*Calymmatobacterium 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; Kubeski *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 9633T (National Collection of Type Cultures)] and *K. rhinoscleromatis* (NCTC 5046T).

Primer sets used in this study include (5'-ACCTACCGCAACACCGACTTCTCCTCGG-3' and 5'-TGAATCAGAACTGGTAGGTCAT-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

<table>
<thead>
<tr>
<th>Organism</th>
<th>16S rRNA (Strain</th>
<th>Accession</th>
<th>phoE (Strain</th>
<th>Accession</th>
</tr>
</thead>
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<tr>
<td>Calymmatobacterium granulomatis</td>
<td>*</td>
<td>AF009171†</td>
<td>*</td>
<td>AF009231†</td>
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<td>NCTC 5046†</td>
<td>AF009169†</td>
<td>NCTC 5046†</td>
<td>AF009230†</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
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<td>X87276</td>
<td>NCTC 9633†</td>
<td>AF064793†</td>
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<td>AB004754</td>
<td>K26</td>
<td>X68022</td>
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<td>Klebsiella ornithinolytica</td>
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<td>AB004756</td>
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<td>DSM 3069†</td>
<td>X93215</td>
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<td>K12</td>
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<td>U88545</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

* Sequences obtained from clinical specimens.
† Sequences novel to this study.
‡ Strain not specified in GenBank report.

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 30104T (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
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*.

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**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.
**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*, *K. 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 bacillary 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 chromatins densities (Rajam & Rangiah, 1954; Sehgal & Sharma, 1992; Hart, 1997). Immature, non-capsulated forms may appear as closed safety pins with certain stains due to bipolar chromatins 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).

**Staining properties.** Gram-negative. Well seen with Giemsa, Leishman, Wright’s or Silver stains. Poorly visualized with hematoxylin and eosin (Richens, 1991). Periodic acid Schiff-negative (Spagnolo et al., 1984; Richens, 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 (Dulany et al., 1948); thioglycollate broth with lactalbumin hydroxylate (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-aerophilic 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 chorionicallantoic 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.

**Acknowledgements**

This study was funded by Territory Health Services, Northern Territory Government. J. S. C. is in receipt of a scholarship from the National Health and Medical Research Council of Australia.

**References**


