NOTE**Phylogenetic position of *Chitinophaga pinensis* in the *Flexibacter*–*Bacteroides*–*Cytophaga* phylum**L. I. Sly,¹ M. Taghavi^{1†} and M. Fegan²

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Comparison of the 16S rRNA gene sequence determined for *Chitinophaga pinensis* showed that this species is most closely related to *Flexibacter filiformis* in the *Flexibacter*–*Bacteroides*–*Cytophaga* phylum. These two chitinolytic bacteria, which are characterized by transformation into spherical bodies on ageing, belong to a strongly supported lineage that also includes *Cytophaga arvensicola*, *Flavobacterium ferrugineum* and *Flexibacter sancti*. The lineage is distinct from the microcyst-forming species *Sporocytophaga myxococcoides*.

Keywords: *Chitinophaga pinensis*, 16S rRNA, phylogeny, bacterial taxonomy, *Flexibacter*–*Bacteroides*–*Cytophaga* phylum

In 1981, Sangkhobol & Skerman described the species *Chitinophaga pinensis* to include five strains of a long, filamentous, chitinolytic, gliding bacterium that transforms on ageing into spherical bodies. The authors likened the coccoid bodies to the microcysts of *Sporocytophaga myxococcoides* (Leadbetter, 1989). Sangkhobol and Skerman noted that the myxospores germinated without any clear evidence of capsule shedding as previously described by Leadbetter (1963) for *Sporocytophaga myxococcoides*, nor did they exhibit the high refractility of *Sporocytophaga myxococcoides* microcysts under phase microscopy. No comparison with other characteristics of *Sporocytophaga myxococcoides* myxospores such as heat and desiccation resistance was made. *Chitinophaga pinensis* was differentiated from *Sporocytophaga myxococcoides* on the bases of cells more than four times the length of *Sporocytophaga myxococcoides*, an ability to hydrolyse chitin but not cellulose, and a G + C content of its genomic DNA of 43–46 mol% compared with 36 mol% for *Sporocytophaga myxococcoides*.

Skerman (1989) later introduced the term microcyst to describe the spherical coccoid bodies but used this term interchangeably with the terms myxospores and resting stage without any clear definitions. Reichenbach (1992) has disputed the formation of microcysts by *Chitinophaga pinensis* and drew attention to the

similarities between the morphology of *Chitinophaga pinensis* and *Flexibacter filiformis* (Reichenbach, 1992).

Current knowledge of the phylogenetic relationships of the flexibacteria derived from 16S rRNA catalogues (Paster *et al.*, 1985) shows that *Flexibacter filiformis* ('*Flexibacter elegans*' strain Fx e1) and *Sporocytophaga myxococcoides* are on phylogenetically distinct lines within the *Flexibacter*–*Bacteroides*–*Cytophaga* phylum.

To date, no 16S rRNA sequence has been available for *Chitinophaga pinensis* to determine its phylogenetic relationship with the other two species of gliding flexibacteria (*Flx. filiformis* and *Sporocytophaga myxococcoides*) reported to form spherical bodies (myxospores, microcysts or resting stages) on ageing. In this study we have sequenced the 16S rDNA of the *Chitinophaga pinensis* type strain ACM 2034^T (= DSM 2588^T) and compared this with the available sequences of *Flexibacter filiformis*, *Sporocytophaga myxococcoides* and other reference sequences in the *Flexibacter*–*Bacteroides*–*Cytophaga* phylum.

The sequencing of the 16S rDNA of *Chitinophaga pinensis* and the phylogenetic analysis was carried out using methods previously described (Sly *et al.*, 1998). Direct PCR amplification of 16S rDNA was performed and the PCR products were purified using Promega Magic PCR Prep DNA Purification according to the manufacturer's instructions. The PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) was used with the primers 27f,

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The GenBank accession number for the rDNA sequence of *Chitinophaga pinensis* ACM 2034^T is AF078775.

Fig. 1. Phylogenetic neighbour-joining dendrogram showing the relationship of *Chitinophaga pinensis* with other related species in the *Flexibacter*-*Bacteroides*-*Cytophaga* phylum. The sequence of *Chlorobium vibrioforme* was used as the outgroup. Bootstrap values of 100 analyses are shown at the branch points. The scale bar represents 10 nucleotide substitutions per 100 nucleotides of 16S rRNA sequence. The strain numbers and sequence accession numbers (in parentheses) of reference sequences used in the analysis are as follows: *Bacteroides fragilis* (M61006); *Chlorobium vibrioforme* DSM 260^T (M62791); *Cyclobacterium marinum* ATCC 43824 (M62788); *Cytophaga arvensicola* IAM 12650^T (D12657); *Cytophaga diffluens* ATCC 23140 (M58765); *Cytophaga hutchinsonii* ATCC 33406^T (M58768); *Empedobacter brevis* ATCC 14234 (M59052); *Flavobacterium aquatile* ATCC 11947^T (M62797); *Flavobacterium ferrugineum* ATCC 13524^T (M62798); *Flectobacillus major* ATCC 29496^T (M62787); *Flexibacter aggregans* ATCC 23162^T (M64628); *Flexibacter canadensis* ATCC 29591^T (M62793); *Flexibacter filiformis* ATCC 29495^T (=Fx e1) (M58782); *Flexibacter flexilis* ATCC 23079^T (M62794); *Flexibacter litoralis* ATCC 23117^T (M58784); *Flexibacter roseolus* ATCC 23088^T (M58787); *Flexibacter ruber* ATCC 23103^T (M58788); *Flexibacter sancti* ATCC 23092^T (M62795); *Flexithrix dorotheae* ATCC 23163^T (AF039296); *Haliscomenobacter hydrossis* ATCC 27775^T (M58790); *Lewinella cohaerens* ATCC 23123^T (AF039262); *Lewinella nigricans* ATCC 23147^T (AF039294); *Lewinella persica* ATCC 23167^T (AF039295); '*Microscilla aggregans* subsp. *catalatica*' ATCC 23190 (M58791); '*Microscilla arenaria*' ATCC 23161 (M60455); '*Microscilla furvescens*', ATCC 23129 (M58792); *Rhodothermus marinus* (X77140); *Runella slithyformis* ATCC 29530^T (M62786); *Saprospira grandis* ATCC 23119^T (M58795); *Spirosoma linguale* ATCC 23276 (M62789); *Sporocytophaga myxococcoides* ATCC 10010^T (spc.myxoco RDP); *Thermonema lapsum* (L11703).

926f, 1114f, 357r, 519r, 907r, 1100r, 1392r and 1525r (Lane, 1991), and 787r and 803f (Stackebrandt & Charfreitag, 1990) to directly sequence the PCR products, following the protocol provided by the manufacturer. The reaction mixtures were sequenced automatically on an Applied Biosystems 373A DNA Sequencer.

The 16S rRNA sequences were aligned manually using the ae2 editor (Maidak *et al.*, 1997) with representative bacterial 16S rRNA gene sequences from GenBank and the rRNA Database Project (Maidak *et al.*, 1997). Positions where length and sequence variations made alignment uncertain were omitted from the analysis. Pairwise evolutionary distances were computed using the method of Jukes & Cantor (1969) in the DNADIST program of the PHYLIP 3.5c software package (Felsenstein, 1993). Phylogenetic dendrograms were constructed using the neighbour-joining method of Saitou & Nei (1987) in PHYLIP. Bootstrap analyses using SEQBOOT from PHYLIP 3.5c were performed to determine the statistical significance of branching patterns and consensus trees (not shown) were produced using CONSENSE (PHYLIP 3.5c).

The dendrogram of relationships (Fig. 1) inferred from a neighbour-joining analysis of the corrected dissimilarity values over a length of 987 nucleotides (restricted by a short 1263 nucleotide sequence for *Cytophaga arvensicola*) indicated that *Chitinophaga pinensis* belongs to a strongly supported lineage in the *Flexibacter*-*Bacteroides*-*Cytophaga* phylum. Members of the lineage also include *Flavobacterium ferrugineum*, *Cytophaga arvensicola*, *Flexibacter sancti*, and its nearest relative, *Flexibacter filiformis*. Nakagawa & Yamasato (1993) have suggested previously that *F. ferruginum*, *Flexibacter sancti* and *Cytophaga arvensicola* may constitute a distinct genus on the bases of their phylogenetic relationship determined from 16S rDNA sequence similarities, their strictly respiratory metabolism, MK-7 menaquinone content and a base composition in the range 42.8–48.6 mol %, which is at the high end of the range for the genus *Flexibacter* (Reichenbach, 1989). Nakagawa & Yamasato (1996) later showed that *Flexibacter filiformis* is also a member of this phylogenetic lineage, but on the basis of bootstrap values showed that the relationship of *Flavobacterium ferrugineum* to the other three members is not strongly supported. The current study has added weight to the emerging taxonomic importance of this deeply branched and well supported lineage which now also includes *Chitinophaga pinensis*. This lineage is well separated from the lineage which contains *Flexibacter flexilis*, the type species of the genus, and supports the transfer of the member species to a new genus. Woese *et al.* (1990) previously reported the widespread distribution of the *Flexibacter* species in the FBC phylum and showed that *Flexibacter flexilis* and *Flexibacter sancti* belonged to widely separated lineages. However, there appear to be few phenotypic characteristics which unify the members of the phylo-type. As mentioned earlier, *Chitinophaga pinensis* and

Flexibacter filiformis are quite similar in morphology and share the ability to degrade chitin. *Cytophaga arvensicola* and *Flexibacter sancti* on the other hand cannot degrade chitin, have much shorter cells and do not form spherical bodies on ageing. In spite of their superficial phenotypic similarity, the 95.3% sequence similarity between the 16S rRNA genes of *Chitinophaga pinensis* and *Flexibacter filiformis* over a length of 1445 nucleotides is indicative of their separate species status (Stackebrandt & Goebel, 1994). The fact that *Sporocytophaga myxococcoides* belongs to a separate phylogenetic lineage in the *Flexibacter-Bacteroides-Cytophaga* phylum as previously reported (Paster *et al.*, 1985), indicates that transformation to spherical bodies is not phylogenetically exclusive and may have been an early evolutionary trait or, more likely, that the nature of the spherical body in *Chitinophaga pinensis* and *Flexibacter filiformis* is different to that in *Sporocytophaga myxococcoides*, as previously suggested by Reichenbach (1992).

References

- Felsenstein, J. (1993). PHYLIP (Phylogeny Inference Package) version 3.5c. Seattle: University of Washington.
- Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.
- Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester: Wiley.
- Leadbetter, E. R. (1963). Growth and morphogenesis of *Sporocytophaga*. *Bacteriol Proc* p. 42.
- Leadbetter, E. R. (1989). Genus IV. *Sporocytophaga* Stanier 1940. In *Bergey's Manual of Systematic Bacteriology*, vol. 3, pp. 2061. Edited by J. T. Staley, M. P. Bryant, N. Pfennig & J. G. Holt. Baltimore: Williams & Wilkins.
- Maidak, B. L., Olsen, G. J., Larsen, N., Overbeek, R., McCaughey, R. & Woese, C. R. (1997). The RDP (Ribosomal Database Project). *Nucleic Acids Res* **25**, 109–111.
- Nakagawa, Y. & Yamasato, K. (1993). Phylogenetic diversity of the genus *Cytophaga* revealed by 16S rRNA sequencing and menaquinone analysis. *J Gen Microbiol* **139**, 1155–1161.
- Nakagawa, Y. & Yamasato, K. (1996). Emendation of the genus *Cytophaga* and transfer of *Cytophaga agarovorans* and *Cytophaga salmonicolor* to *Marinilabilia* gen. nov.: phylogenetic analysis of the *Flavobacterium-Cytophaga* complex. *Int J Syst Bacteriol* **46**, 599–603.
- Paster, B. J., Ludwig, W., Weisburg, W. G., Stackebrandt, E., Hespell, R. B., Hahn, C. M., Reichenbach, H., Stetter, K. O. & Woese, C. R. (1985). A phylogenetic grouping of the bacteroides, cytophagas, and certain flavobacteria. *Syst Appl Microbiol* **6**, 34–42.
- Reichenbach, H. (1989). Genus *Flexibacter* Soriano 1945, 92^{AL} emend. In *Bergey's Manual of Systematic Bacteriology*, vol. 3, pp. 2061–2071. Edited by J. T. Staley, M. P. Bryant, N. Pfennig & J. G. Holt. Baltimore: Williams & Wilkins.
- Reichenbach, H. (1992). The order *Cytophagales*. In *The Prokaryotes*, 2nd edn, vol. IV, pp. 3631–3675. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer.
- Saitou, N. & Nei, M. (1987). The neighbour-joining method: a new method for constructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sangkhobol, V. & Skerman, V. B. D. (1981). *Chitinophaga*, a new genus of chitinolytic myxobacteria. *Int J Syst Bacteriol* **31**, 285–293.
- Skerman, V. B. D. (1989). Genus *Chitinophaga* Sangkhobol and Skerman 1981. In *Bergey's Manual of Systematic Bacteriology*, vol. 3, pp. 2074–2077. Edited by J. T. Staley, M. P. Bryant, N. Pfennig & J. G. Holt. Baltimore: Williams & Wilkins.
- Sly, L. I., Taghavi, M. & Fegan, M. (1998). Phylogenetic heterogeneity within the genus *Herpetosiphon*: transfer of the marine species *Herpetosiphon cohaerens*, *Herpetosiphon nigricans*, and *Herpetosiphon persicus* to the genus *Lewinella* gen. nov. in the *Flexibacter-Bacteroides-Cytophaga* phylum. *Int J Syst Bacteriol* **48**, 731–737.
- Stackebrandt, E. & Charfreitag, O. (1990). Partial 16S rRNA primary structure of five *Actinomyces* species: phylogenetic implications and development of an *Actinomyces israelii*-specific oligonucleotide probe. *J Gen Microbiol* **136**, 37–43.
- Stackebrandt, E. & Goebel, B. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.
- Woese, C. R., Yang, D., Mandelco, L. & Stetter, K. O. (1990). The flexibacter-flavobacter connection. *Syst Appl Microbiol* **13**, 161–165.