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NOTE

Phylogenetic characterization of a novel radiation-resistant bacterium from irradiated pork: description of *Hymenobacter actinosclerus* sp. nov.

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A phylogenetic analysis was performed on a red-pigmented, radiation-resistant, Gram-negative, rod-shaped organism originating from irradiated pork. Comparative 16S rRNA gene sequencing showed the bacterium was a member of the *Cytophaga–Flavobacterium–Bacteroides* line of descent and represents a new subline within the genus *Hymenobacter*. A new species, *Hymenobacter actinosclerus*, is described for this novel radiation-resistant bacterium. The type strain of *Hymenobacter actinosclerus* is CCUG 39621^T.

Keywords: *Hymenobacter actinosclerus* sp. nov., taxonomy, phylogeny, 16S rRNA, radiation-resistant bacterium

To date relatively few radiotolerant bacteria have been described. The majority of recognized radiation-resistant bacteria are members of the genus *Deinococcus*. The genus *Deinococcus* was created for '*Micrococcus radiodurans*' and some other red-pigmented, radiation-resistant cocci, and five species (viz. *Deinococcus erythromyxa*, *Deinococcus proteolyticus*, *Deinococcus radioduans*, *Deinococcus radiophilus* and *Deinococcus radiopugnans*) were assigned to the genus by Brooks & Murray (1981). With the exception of *D. erythromyxa*, which has been shown to be related to the Actinomycete division of the Gram-positive bacteria and is a member of the genus *Kocuria*, other deinococci form a coherent phylogenetic cluster related to the *Thermus–Meiothermus* lineage (Rainey *et al.*, 1997). *Deinobacter grandis* is another radiation-resistant bacterium, which phenotypically resembles deinococci but was assigned to a separate genus on the basis of its rod-shaped cellular morphology (Oyaizu *et al.*, 1987). Phylogenetic studies have, however, shown *Deinobacter grandis* falls within the *Deinococcus* clade, and this species has subsequently been reclassified (Rainey *et al.*, 1997). Another notable radiation-resistant organism is *Rubrobacter* (formerly *Arthrobacter*) *radiotolerans*, a thermotolerant, pink-pigmented bacterium isolated from a radon-containing hot spring (Yoshinaka *et al.*, 1973). A second species of this genus, *Rubrobacter xylanophilus*, was described by Carreto *et al.* (1996) from a thermally polluted industrial effluent. Phylogenetically, rubrobacters are related to the high-G+C actinomycete branch of the Gram-positive bacteria. In an earlier study, we reported the isolation of a red-pigmented, radiation-resistant, Gram-negative rod-shaped organism from irradiated pork (Grant & Patterson, 1989). The observed levels of radiation resistance (D_{10} values in buffer solution and on pork mince of 3·45 and 5·05 kGy, respectively), were similar to those of *Deinococcus* (formerly *Deinobacter*) *grandis* and other deinococci. The cell wall of the rod-shaped bacterium from irradiated pork possessed a layered structure typical of Gram-negative bacteria, but the looped external membranous structure described by Oyaizu *et al.* (1987) for *D. grandis* was not present (Grant & Patterson, 1989). The very considerable phylogenetic diversity of currently described radiation-resistant bacteria has prompted us to investigate the evolutionary relationships of the curious rod-shaped bacterium from pork. Based on these phylogenetic findings and the results of an earlier study (Grant & Patterson, 1989), we propose the radiation-resistant rod from irradiated pork be classified as a new species of the genus *Hymenobacter*, *Hymenobacter actinosclerus* sp. nov.

The isolate from pork has been deposited in the Culture Collection of the University of Göteborg (CCUG), Sweden under accession number CCUG 39621^T. Traditional biochemical and physiological tests were performed as described previously (Grant & Patterson, 1989). API ZYM enzyme substrate strips

The GenBank accession number for the 16S rRNA gene sequences of *Hymenobacter actinosclerus* strain CCUG 39621^T is Y17356.

(API bioMérieux) were used to determine enzymic activities. Cells for wall murein, long-chain cellular fatty acid, menaquinone and DNA G+C content determination were grown in yeast extract peptone (YP) broth for 36–48 h at 25 °C. DNA was prepared according to the method of Marmur (1961) and DNA G+C content was determined by thermal denaturation, using a Gilford model 250 spectrophotometer. The G+C content was calculated as described by Johnson (1981). The diamino acid content of the cell wall murein was determined by the method of Schleifer & Kandler (1972). Fatty acid methyl esters were prepared and analysed as described by Kämpfer & Kroppenstedt (1996). Menaquinone composition was determined as described by Kroppenstedt (1982). The 16S rRNA gene sequence of the isolate was determined by PCR direct sequencing. Sequencing was performed using a Taq DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 363A; Applied Biosystems). The closest known relatives of the isolate were determined by performing database searches. These sequences and those of other known related strains were retrieved from GenBank or Ribosomal Database Project libraries and aligned with the newly determined sequence using the program PILEUP (Devereux *et al*., 1984). The resulting multiple alignment was corrected manually and a distance matrix was calculated using the program PRETTY from DNADIST (using the Kimura 2-correction parameter) (Felsenstein, 1989). A phylogenetic tree was constructed according to the neighbour-joining method with the program NEIGHBOR (Felsenstein, 1989). The stability of the groupings was estimated by bootstrap analysis (200 replications) using the programs DNABOOT, DNADIST, NEIGHBOR and CONSENSE (Felsenstein, 1989). In addition, parsimony analysis was performed on the same data set (Felsenstein, 1989).

Consistent with an earlier report (Grant & Patterson, 1989), the unknown rod-shaped bacterium from irradiated pork was aerobic, and oxidase- and catalase-positive. The organism did not produce acid or gas from glucose and failed to assimilate L-arabinose, D-mannose, D-mannitol, maltose, D-gluconate, sucrose or trehalose. It hydrolysed starch but not aesculin. It was indole-negative, did not produce H_2S and failed to reduce nitrate. Using the API ZYM system, activities for alkaline phosphatase, acid phosphatase, ester lipase C8, cystine arylamidase, leucine arylamidase, valine arylamidase and phosphoamidase were detected. No activity was detected for lipase C14, chymotrypsin, trypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, α-fucosidase, α-mannosidase or urease. Grant & Patterson (1989) in an earlier study reported the bacterium stained Gram-negative and its wall possessed a layered structure typical of Gram-negative bacteria. An examination of the wall murein amino acid composition in the present study revealed *meso*-diaminopimelic acid as the dibasic amino acid. This result is consistent with the presence of a directly cross-linked *meso*-diaminopimelic acid (variation A1γ)-type murein in the cell wall of the unknown rod. This murein type is present in most Gram-negative taxa but contrasts markedly with that reported for deinococci (including *Deinobacter grandis*) which contain L-ornithine as the dibasic amino acid. The long-chain cellular fatty acid composition of the unidentified rod also differed from that of deinococci. The major fatty acids of the bacterium were of the iso-/anteiso-methyl branched and hydroxy iso-methyl branched types (major components anteiso-$C_{15:0}$, 23%; iso-$C_{15:0}$, 22%; $C_{16:1}$ plus 2 OH-iso-$C_{15:0}$, 22% summed feature; anteiso-$C_{17:1}$, 9%; 3 OH-iso-$C_{17:0}$, 3%) and resemble those of the *Cytophaga–Flavobacterium* phylum. By contrast, deinococci contain predominantly iso-methyl branched and monounsaturated acids and lack hydroxy acids (Oyaizu *et al*., 1987). The unidentified organism also differed from deinococci in menaquinone composition. MK-7 was the sole menaquinone detected in strain CCUG 39621^T whereas deinococci invariably contain MK-8 as the predominant respiratory component.

To ascertain the phylogenetic position of the unknown bacterium, its 16S rRNA gene sequence was amplified by PCR and sequenced directly. Comparative sequence analysis revealed the bacterium was only remotely related to deinococci and other described radiation-resistant organisms (< 77% 16S rRNA sequence similarity). Sequence searches of GenBank revealed the unknown radiation-resistant rod was a member of the *Cytophaga–Flavobacterium* phylum and exhibited highest sequence relatedness with *Hymenobacter roseosalivarius* (94·4%) and '*Taxeobacter*' spp. (91–95%). A tree depicting the phylogenetic relationships of the unknown organism constructed using the neighbour-joining method is shown in Fig. 1 and clearly demonstrates its close association with *H. roseosalivarius* and 'taxeobacters'. Bootstrap resampling, however, showed there was no particularly significant affinity between the unknown bacterium and any of these organisms. Parsimony analysis was also conducted on the same data set and confirmed the results of neighbour-joining.

It is evident from the findings of the present polyphasic taxonomic study that the unknown Gram-negative, rod-shaped organism from irradiated pork is distinct from all other currently described radiation-resistant taxa. The phenotypic characteristics (including chemical biomarkers) of the bacterium are strongly indicative of an affinity with members of the *Cytophaga–Flavobacterium* group of organisms, an observation consistent with 16S rRNA gene sequence analysis. Phylogenetically, the unknown bacterium is closely associated with *Hymenobacter* and 'taxeobacters'. The genus *Hymenobacter* currently contains a single species, *H. roseosalivarius* and was described by Hirsch *et al*. (1998) to accommodate a group of red/pink-pigmented rods originating from Antarctic soils and sandstone. Hirsch *et al*. (1998) showed a group of red/pink-pigmented, spreading bacteria,

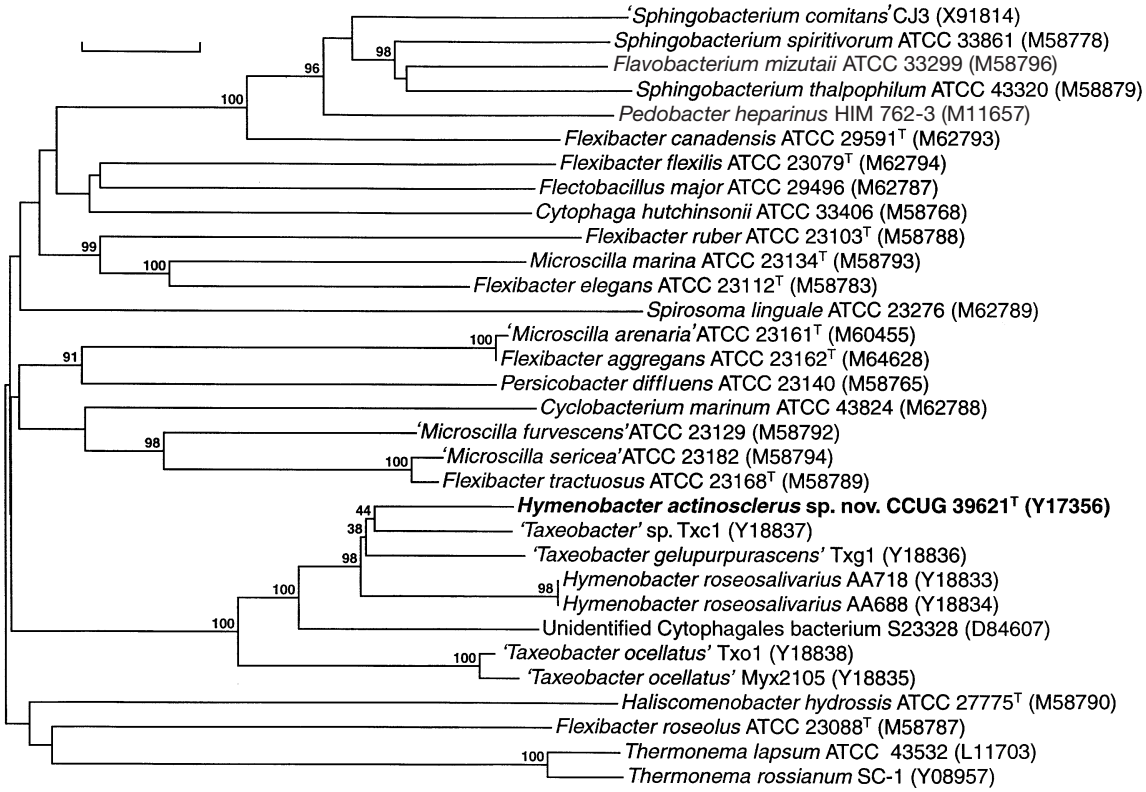

Fig. 1. Unrooted tree showing the phylogenetic position of *Hymenobacter actinosclerus*. The tree was constructed using the neighbour-joining method and was based on a comparison of approximately 1320 nucleotides. Bootstrap values, expressed as a percentage of 200 replications, are given at branching points. The scale bar indicates 2 % divergence.

which have been not validly described and which were tentatively named 'taxeobacters' by Reichenbach (1992), to be the closest phylogenetic relatives of this new genus. It is evident from the present study that the unidentified red/pink-pigmented, radiation-resistant bacterium from pork forms a robust group with *H. roseosalivarius* and two 'taxeobacters' designated '*Taxeobacter gelupurpurascens*' Txg1 and '*Taxeobacter*' sp. Txc1. The overall clustering together of these organisms was found to be highly significant (as indicated by a bootstrap resampling value of 98 %), which, combined with sequence divergence values of approximately 5 %, indicates these merit classification as a single genus. Two other organisms designated '*Taxeobacter ocellatus*' and an unidentified Cytophagales bacterium also clustered with the aforementioned taxa (Fig. 1), although sequence divergence values of approximately 8 % suggest their affinity is probably at a suprageneric level. Based upon phylogenetic evidence, we therefore consider the unknown radiation-resistant bacterium CCUG 39621^T represents a hitherto unknown species of the genus *Hymenobacter*, for which the name *Hymenobacter actinosclerus* sp. nov. is proposed. Radiation-resistant bacteria, such as *D. radiodurans*, are known to occur naturally in foods. The present study shows the occurrence of a highly radiation-resistant bacterium from a quite separate taxonomic lineage (i.e. *Cytophaga–Flavobacterium–Bacteroides* line of descent) in foods. This organism adds to the considerable phylogenetic diversity of currently described radiation-resistant bacteria. The significance of high-level radiation resistance within the novel *Hymenobacter* species from pork is not known but if low-dose irradiation were to be used on fresh meats (e.g. to extend shelf-life) this organism, and possibly others, would not be killed. It is also pertinent to note that no information is available on the sensitivity of other *Hymenobacter* species and 'taxeobacters' to ionizing radiation.

Description of *Hymenobacter actinosclerus* sp. nov.

Hymenobacter actinosclerus (ac.ti.no.scle′rus. Gr. n. *actis*, *actinos* ray, beam; Gr. adj. *scleros* hard; M.L. masc. n. *actinoscleros* hard against rays, pertaining to the organism's radiation resistance).

Cells are Gram-negative rods, ranging from 0·5 to 0·6 μm in width and 2·0 to 3·6 μm in length, which are non-motile in hanging drops. No variation in cell morphology is observed in old cultures. Colonies on YP agar are circular, entire, opaque and not easily emulsified. Chemo-organotrophic (metabolism respiratory). Water-insoluble red pigment is produced. Fluorescent pigments are not produced on King's A or

B media. Aerobic, with ability to grow under microaerobic conditions. Oxidase- and catalase-positive. Acid and gas are not produced from D-glucose. Starch is hydrolysed, but aesculin is not. Using the API ZYM system, alkaline phosphatase, acid phosphatase, ester lipase C8, cystine arylamidase, leucine arylamidase, valine arylamidase, *N*-acetyl-β-glucosaminidase and phosphoamidase activity are detectable. Lipase C14, chymotrypsin, trypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, α-fucosidase, α-mannosidase and urease activity are not detectable. Nitrate reduction, H_2S production and indole production are negative. Growth occurs at 42 °C but not at 5 °C. Optimal temperature for growth is 25–30 °C. Highly radiation-resistant with D_{10} in sodium phosphate buffer of 3·45 kGy and on minced pork of 5·05 kGy. *meso*-Diaminopimelic acid is present in the cell wall murein. The major respiratory quinone is MK-7. The major cellular fatty acids are of the iso-/anteiso-methyl branched and hydroxy iso-methyl branched types. DNA base composition is 62 mol% G+C (T_m). Isolated from pork chops irradiated to a dose of 1·75 kGy. The type strain is CCUG 39621^T.

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References

Brooks, B. W. & Murray, R. G. E. (1981). Nomenclature for '*Micrococcus radiodurans*' and other radiation-resistant cocci: *Deinococcaceae* fam. nov. and *Deinococcus* gen. nov., including five species. *Int J Syst Bacteriol* **31**, 353–360.

Carreto, L., Moore, E., Nobre, M. F., Wait, R., Riley, P. W., Sharp, R. J. & Da Costa, M. S. (1996). *Rubrobacter xylanophilus* sp. nov., a new thermophilic species isolated from a thermally polluted effluent. *Int J Syst Bacteriol* **46**, 460–465.

Devereux, J., Haeberli, P. & Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* **12**, 387–395.

Felsenstein, J. (1989). PHYLIP – phylogeny inference package (version 3.2). *Cladistics* **5**, 164–166.

Grant, I. R. & Patterson, M. F. (1989). A novel radiation-resistant *Deinobacter* sp. isolated from irradiated pork. *Lett Appl Microbiol* **8**, 21–24.

Hirsch, P., Ludwig, W., Hethke, C., Sittig, M., Hoffmann, B. & Gallikowski, C. A. (1998). *Hymenobacter roseosalivarius* gen. nov., sp. nov. from continental Antarctic soils and sandstone: bacteria of the *Cytophaga/Flavobacterium/Bacteroides* line of phylogenetic descent. *Syst Appl Microbiol* **21**, 374–383.

Johnson, J. L. (1981). Genetic characterization. In *Manual of Methods for General Bacteriology*, pp. 450–472. Edited by P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg & G. B. Phillips. Washington, DC: American Society for Microbiology.

Kämpfer, P. & Kroppenstedt, R. M. (1996). Numerical analysis of fatty acid patterns of coryneform bacteria and related taxa. *Can J Microbiol* **42**, 989–1005.

Kroppenstedt, R. M. (1982). Separation of bacterial menaquinones by HPLC using reverse phase (RP18) and a silver loaded ion exchanger as stationary phases. *J Liq Chromatogr* **5**, 2359–2367.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.

Oyaizu, H., Stackebrandt, E., Schleifer, K. H., Ludwig, W., Pohla, H., Ito, H., Hirata, A., Oyaizu, Y. & Komagata, K. (1987). A radiation-resistant rod-shaped bacterium, *Deinobacter grandis* gen. nov., sp. nov., with peptidoglycan containing ornithine. *Int J Syst Bacteriol* **37**, 62–67.

Rainey, F. A., Nobre, M. F., Schumann, P., Stackebrandt, E. & Da Costa, M. S. (1997). Phylogenetic diversity of deinococci as determined by 16S ribosomal DNA sequence comparison. *Int J Syst Bacteriol* **47**, 510–514.

Reichenbach, H. (1992). *Taxeobacter*, a new genus of the *Cytophagales* with three new species. In *Advances in the Taxonomy and Significance of Flavobacterium*, *Cytophaga*, *and Related Bacteria*, pp. 182–185. Edited by P. J. Joste. Bloemfontein, Republic of South Africa: University Press.

Schleifer, K. H. & Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* **34**, 407–477.

Yoshinaka, T., Yano, K. & Yamaguchi, H. (1973). Isolation of a highly radioresistant bacterium, *Arthrobacter radiotolerans* nov. sp. *Agric Biol Chem* **37**, 2269–2275.