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Schlegelella thermodepolymerans gen. nov., sp. nov., a novel thermophilic bacterium that degrades poly(3-hydroxybutyrate-*co*-3-mercaptopropionate)

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A novel thermophilic bacterium, strain K14^T, capable of degrading poly(3-hydroxybutyrate) as well as copolymers containing 3-hydroxybutyrate and 3-mercaptopropionate linked by thioester bonds, was isolated. 16S rDNA sequence analysis showed that strain DhA-71, a dehydroabietic acid-degrading bacterium, was the nearest phylogenetic neighbour and that both strains should be placed as members of a newly created genus, *Schlegelella* gen. nov., in the *Rubrivivax* subgroup of the *β-Proteobacteria*. Strain K14^T (=LMG 21644^T=DSM 15344^T) is proposed as the type strain of *Schlegelella thermodepolymerans* gen. nov., sp. nov. Its phylogenetic, morphological, biochemical and chemotaxonomic characteristics are described in detail.

Polyhydroxyalkanoates (PHAs) such as poly(3-hydroxybutyrate) [poly(3HB)] and other PHAs are accumulated by a wide range of bacteria as intracellular storage compounds under conditions of nutrient stress, e.g. when an external carbon source is available but the concentration of another nutrient is limiting growth. Owing to their properties as biodegradable, non-toxic thermoplastics and/or elastomers, these biopolyesters have attracted much interest and have been considered for various technical applications (Hocking & Marchessault, 1994). Degradation of PHA has been investigated in different natural environments (Mergaert & Swings, 1996). PHA-degrading micro-organisms excrete specific extracellular PHA depolymerases that degrade PHAs and utilize the degradation products as carbon and energy sources for growth (Delafield *et al.*, 1965). Many studies have been carried out with mesophilic polymer-degrading bacteria, but relatively few with thermophilic bacteria. Purification of a PHA depolymerase exhibiting optimum activity at 70 °C from the culture broth of *Comamonas testosteroni* ATSU has been described (Kasuya *et al.*, 1994) but, generally, little information is available about thermophilic poly(3HB) degradation (Takeda *et al.*, 1998).

Recently, polymers with a completely different linkage type were identified in the Gram-negative PHA-accumulating bacterium *Ralstonia eutropha*. In addition to 3HB, these polymers contained 3-mercaptopropionate (3MP) or 3-mercaptobutyrate (3MB) (Lütke-Eversloh *et al.*, 2001a, b). The peculiarity of poly(3HB-*co*-3MP) and poly(3HB-*co*-3MB) was the occurrence of thioester linkages in the polymer backbone. Therefore, they represented members of a novel class of biopolymers that were referred to as polythioesters.

In this study, we have isolated the thermophilic polymer-degrading bacterial strain K14^T, which was able to use poly(3HB-*co*-3MP) as a carbon source for growth. 16S rDNA sequence analysis of strain K14^T indicated that this novel isolate was related phylogenetically to the resin acid-degrading thermophilic bacterium DhA-71, isolated by Yu & Mohn (1999), which is capable of degrading dehydroabietic acid at high temperatures. Resin acids are tricyclic diterpenes that occur in many trees and dominate in conifers and, hence, occur in pulp and mill effluents. In general, such resin acids are removed efficiently by biological treatment systems (Kahmark & Unwin, 1996; Liss *et al.*, 1997). Based on phenotypic, chemotaxonomic and phylogenetic results for strains K14^T and DhA-71, we describe a novel species belonging to a new genus, for which we propose the name *Schlegelella thermodepolymerans* gen. nov., sp. nov.

Strain K14^T was isolated from activated sludge under aerobic and thermophilic conditions. The enrichment culture was prepared by inoculating 5 ml sample of activated sludge from Fayoum, Egypt, in 50 ml mineral salts medium (MSM) broth (Schlegel *et al.*, 1961) supplemented with

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Abbreviations: 3HB, 3-hydroxybutyrate; 3MB, 3-mercaptobutyrate; 3MP, 3-mercaptopropionate; PHA, polyhydroxyalkanoate.

The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of strain K14^T is AY152824.

0·15 % (w/v) poly(3HB) or poly(3HB-*co*-3MP) as sole carbon and energy source and incubating the cultures at 55 °C for 1 week. The polymers were prepared according to Jendrossek *et al.* (1993). Serial dilutions of the enrichment culture were spread on poly(3HB-*co*-3MP)-overlay MSM agar plates. Isolate K14^{T} was chosen for its ability to form clear zones. To enhance growth of strain K14^{T}, MSM was supplemented with the following vitamins (l^{-1}): 20 µg biotin, 20 µg folic acid, 60 µg lipoic acid, 50 µg thiamin, 50 µg riboflavin, 50 µg nicotinic acid, 100 µg pyridoxal hydrochloride, 50 µg pantothenic acid, 50 µg vitamin B_{12}, 50 µg *p*-aminobenzoic acid and 200 µg naphthoquinone (Mohn, 1995).

Genomic DNA extraction, PCR amplification of the 16S rDNA and purification of PCR products were carried out using procedures described previously (Rainey *et al.*, 1996). The PCR product was purified by using a Nucleotrap PCR extraction kit (Macherey-Nagel). The following oligonucleotides were used as primers: 27f (5′-GAGTTTGATCCTGGCTCAG-3′), 343r (5′-CTGCTGCCTCCCGTA-3′), 357f (5′-TACGGGAGGCAGCAG-3′), 519r [5′-G(T/A)ATTACCGCGGC(T/G)GCTG-3′], 536f [5′-CAGC(C/A)GCCGCGCGGTAAT(T/A)C-3′], 803f (5′-ATTAGATACCCTAGGTAG-3′), 907r (5′-CCGTCAATTCATTTGAGTTT-3′), 1114f (5′-GCAACGAGCGCAACCC-3′), 1385r [5′-CGGTGTGT(A/G)CAAGGCCC-3′] and 1525r (5′-AGAAAGGAGGTGATCCAGCC-3′).

Sequencing reactions were performed using a SeqiTherm Long-Read cycle sequencing kit (Epicentre Technologies) in a model 4000L semiautomatic DNA sequencer (LI-COR). The 16S rDNA sequences were initially analysed by using the program BLAST (National Center for Biotechnology Information; http://www.ncbi.nml.nih.gov). The consensus sequence of strain K14^{T} and sequences of strains belonging to the same phylogenetic group and of other representatives of the *β*-subclass of the *Proteobacteria* (retrieved from the EMBL database) were aligned using CLUSTAL X (Thompson *et al.*, 1997). The resulting trees were displayed with TreeView (Page, 1996). The phylogenetic tree was calculated using the neighbour-joining method (Saitou & Nei, 1987), and the neighbour-joining tree was inferred by using TreeCon with *Ralstonia metallidurans* as an outgroup.

The 16S rDNA sequence data indicated that strain K14^{T} is related to the dehydroabietic acid-degrading bacterium DhA-71, described by Mohn (1995). The 16S rDNA sequence of strain K14^{T} revealed similarity values to its nearest phylogenetic neighbours of 99 % (strain DhA-71) and 93 % (*Leptothrix* sp. oral clone AW043). These results indicate that strains K14^{T} and DhA-71 belong to a new thermophilic genus in the *Rubrivivax* subgroup of the *β*-subclass of the *Proteobacteria*. This is also evident from the neighbour-joining dendrogram (Fig. 1).

To determine the G+C content, DNA was prepared according to the method of Pitcher *et al.* (1989) from cells grown on TSA medium (Difco) at 50 °C. The DNA base composition was determined using an HPLC method. DNA was enzymically degraded into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture obtained was then separated by HPLC using a Waters Symmetry Shield C8 column maintained at 37 °C. The solvent was 0·02 M $NH_4H_2PO_4$, pH 4·0, in 1·5 % acetonitrile. Non-methylated lambda phage DNA (Sigma) was used as the calibration reference. The G+C content of strains K14^{T} and DhA-71 was 70·0 mol%.

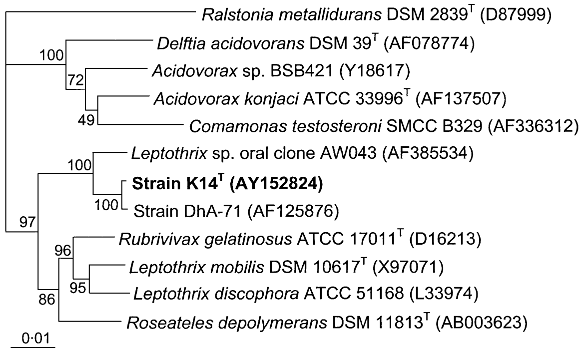

Fig. 1. Neighbour-joining tree based on 16S rDNA sequences showing the estimated phylogenetic relationships of strain K14^{T} and the nearest members of the *β-Proteobacteria*. Accession numbers are given in parentheses. Bootstrap values are shown as percentages of 1000 replicates. Bar, 1 % sequence divergence.

DNA–DNA hybridizations were carried out with photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989), using an HTS7000 BioAssay Reader (Perkin Elmer) measuring fluorescence. The hybridization temperature was 50 °C. Reciprocal experiments were performed for every pair of strains. DNA–DNA hybridization confirmed that strains K14^{T} and DhA-71 are closely related, with a DNA reassociation level of 94 %, and thus constitute a single species according to the recommendations of Wayne *et al.* (1987).

Gram-staining was performed according to Gerhardt *et al.* (1994). Wet mounts for motility determination from various growth stages were prepared from liquid broth and also from solid media and were examined with an Ortholux II microscope (Leitz). Flagella staining was performed using the method of Heimbrook *et al.* (1989). Cells of strain K14^{T} were Gram-negative rods and motile by means of one polar flagellum. The dimensions of the cells were 0·5–0·6 × 1·0–2·8 µm when grown in sodium gluconate MSM. Cells of strain DhA-71 were rod-shaped, motile cells, 0·6 × 1·8 µm when grown in dehydroabietic acid-containing MSM (Yu & Mohn, 1999).

Strains K14^{T} and DhA-71 exhibited some similarity in colony morphology, such as being white and smooth and exhibiting convex elevation, entire margins and butyrous texture, but they were different with regard to colony form and opacity: whereas colonies of K14^{T} were punctiform and opaque, those of strain DhA-71 were circular and translucent (Yu & Mohn, 1999).

For fatty acid analysis, the strains were grown for 48 h at 50 °C on TSA medium. Fatty acids were methylated and extracted and separated by GLC using the MIDI system (Microbial ID) as described before (Mergaert *et al.*, 1993). Strains K14^T and DhA-71 showed very similar fatty acid profiles. Their mean composition was: 1·3 % 10 : 0, 3·3 % 10 : 0 3-OH, 1·6 % 12 : 0, 2·0 % 12 : 0 3-OH, 43·1 % 16 : 0, 32·6 % 17 : 0 cyclo, 4·4 % 18 : 1ω7*c* and 2·2 % of other fatty acids (each accounting for less than 1 %).

Growth of the strains was investigated in liquid MSM and on MSM agar plates and utilization of carbon sources was determined according to Schirmer *et al.* (1995) and Gerhardt *et al.* (1994). The dehydroabietic acid-degrading bacterium DhA-71 was cultivated as described by Mohn (1995). Utilization of resin acid was determined according to Yu & Mohn (1999). Utilization of 3′,3′-thiodipropionic acid or 3-mercaptopropionic acid was tested by using these compounds at respective concentrations of 0·2 % and 0·15 % (w/v). Activities of catalase, oxidase, lipase and urease were estimated according to Gerhardt *et al.* (1994). Hydrolysis of gelatin, starch and lipids, indole formation from tryptone, ammonia formation from arginine, nitrate reduction, citrate utilization and hydrogen sulfide production were investigated according to Gerhardt *et al.* (1994) and Harrigan & McCance (1966). Test substrates were added to MSM and the cultures were incubated at 50 °C.

The susceptibility of the strains towards antibiotics was determined according to Schirmer *et al.* (1995) on MSM agar plates for the following antibiotics (ml^{-1}): 15 μg tetracycline, 100 μg streptomycin, 50 μg kanamycin, 50 μg ampicillin and 34 μg chloramphenicol.

Common biochemical and physiological characteristics were examined with cells of strains K14^T and DhA-71 cultivated in liquid MSM microtitre plates and are given below in the descriptions of the genus and species. In addition, the strains grow well on Luria–Bertani and nutrient broth plates at 37, 45 and 55 °C and at pH 6, 7 and 8. They were positive (mainly strain K14^T) or weakly positive (mainly strain DhA-71) for utilization of inositol, citrate and succinate, urease, catalase, growth at 60 °C and growth at pH 9. Strains K14^T and DhA-71 were negative for growth at 4 °C and at pH 10. Strain K14^T differed from strain DhA-71 in its ability to utilize arabinose, glucose, starch, pyruvate, acetate, palmitate, arachidate and poly (3HB-*co*-3MP), to produce hydrogen sulfate from cysteine and to reduce nitrate to nitrogen and its inability to grow at pH 5·5. In addition, strain K14^T grew weakly on galactose, fructose, sucrose, trehalose, oleate, 3′,3′-thiodipropionate and 3-mercaptopropionate, while strain DhA-71 did not utilize these substrates but grew at 30 °C and pH 5.

The phylogenetic position of strains K14^T and DhA-71 and their thermophily support their accommodation in a new genus. We propose to classify them in the same species on the basis of their high genomic relatedness, supported by their very similar fatty acid profiles, and propose the name *Schlegelella thermodepolymerans* gen. nov., sp. nov. In contrast to the nearest related genus, *Leptothrix* (Spring *et al.*, 1996), *S. thermodepolymerans* does not produce pigments, grows at 45 and 55 °C and utilizes citrate, succinate and gluconate. Furthermore, total fatty acid extracts of *S. thermodepolymerans* are characterized by large amounts of cyclic fatty acids, which are absent in extracts from *Leptothrix* strains (Spring *et al.*, 1996) and rare in other members of the *β*-*Proteobacteria* (Willems *et al.*, 1990).

Description of *Schlegelella* gen. nov.

Schlegelella (Schle.gel.el′la. L. fem. dim. ending -*ella*; N.L. fem. n. *Schlegelella* named in honour of H. G. Schlegel, a pioneer in PHA research).

Gram-negative, non-spore-forming aerobic rods. The temperature range for growth is 37–60 °C, with an optimum around 50 °C. Citrate, succinate and gluconate are utilized. Cells are oxidase- and catalase-positive. The predominant fatty acids are 16 : 0 and 17 : 0 cyclo. The genus belongs phylogenetically to the *β*-subclass of the *Proteobacteria*, with *Schlegelella thermodepolymerans* as the type species.

Description of *Schlegelella thermodepolymerans* sp. nov.

Schlegelella thermodepolymerans [ther′mo.de.po.ly′me.rans. Gr. n. *therme* heat; N.L. v. *depolymerare* to depolymerize; N.L. part. adj. *thermodepolymerans* depolymerizing in the heat, referring to the ability to degrade poly(3-hydroxybutyrate) at high temperatures].

Description is as for the genus. Additional characteristics are based on data obtained for strains K14^T and DhA-71. Cells are 1·0–2·8 μm long and 0·5–0·6 μm wide and are motile by means of polar monotrichous flagellation. Strains grow well on complex media at 45–50 °C. Colonies are white and smooth, with a convex elevation, entire margins and butyrous texture, either opaque or translucent. pH range for growth is 6–9 with an optimum at pH 7. Acid is not produced from glucose. Strains utilize dehydroabietic acid, gluconate, lactate, 3-hydroxybutyrate, valerate and poly(3-HB) but not xylose, mannose, hexanoate, octanoate, benzoate, ethanol or poly(3-hydroxyoctanoate). Susceptible to tetracycline, kanamycin and chloramphenicol and resistant to streptomycin and ampicillin. Aesculin and gelatin are hydrolysed. Indole is not produced. Arginine dihydrolase-positive. The G + C content of the DNA is 70·0 mol%.

The type strain, strain K14^T (= LMG 21644^T = DSM 15344^T), was isolated from an activated sludge sample after enrichment on poly(3HB-*co*-3MP) as sole carbon source. A second strain of the species is strain DhA-71 (= LMG 21645), isolated by Yu & Mohn (1999) from paper mill effluent.

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