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Malikia granosa gen. nov., sp. nov., a novel polyhydroxyalkanoate- and polyphosphate-accumulating bacterium isolated from activated sludge, and reclassification of *Pseudomonas spinosa* as *Malikia spinosa* comb. nov.

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A Gram-negative, motile, rod-shaped bacterium, designated strain P1^T, was isolated from activated sludge of a municipal wastewater treatment plant. Phylogenetic analysis of its 16S rRNA gene sequence placed the novel isolate among representatives of the family *Comamonadaceae*. The closest relatives in reconstructed phylogenetic trees were *Pseudomonas spinosa*, *Macromonas bipunctata* and *Hydrogenophaga* species. Strain P1^T was not able to grow anaerobically or autotrophically, reduced nitrate to nitrite and required vitamins for growth. Ubiquinone 8 (Q8) and 3-hydroxy-substituted fatty acids were present, but 2-hydroxy fatty acids were absent. The G + C content of the DNA was 67 mol%. Phenotypic characteristics allowed a clear differentiation of strain P1^T from representatives of the genera *Hydrogenophaga* and *Macromonas*, whereas DNA–DNA hybridization experiments revealed that strain P1^T did not belong to the species *P. spinosa*. As a peculiarity, cells of strain P1^T and *P. spinosa* ATCC 14606^T were able to accumulate large amounts of polyhydroxyalkanoates and polyphosphate in the form of large intracellular granules. Apparently in both strains nitrogen limitation stimulates the production of polyhydroxyalkanoates, whereas carbon starvation induces the formation of polyphosphates. Based upon phylogenetic and phenotypic evidence, it is proposed to establish the novel taxon *Malikia granosa* gen. nov., sp. nov., represented by the type strain P1^T (=DSMZ 15619^T=JCM 12706^T=CIP 108194^T). The most closely related species of strain P1^T was *P. spinosa*. This species has been misclassified, and it is proposed to transfer it to the new genus *Malikia* as *Malikia spinosa* gen. nov., comb. nov. The type strain is ATCC 14606^T (=DSMZ 15801^T).

Since 1993 several studies applying cultivation-independent techniques have demonstrated that members of the β -*Proteobacteria*, especially the family *Comamonadaceae*, may represent a major fraction of the metabolically active

microbial population in activated sludge of municipal sewage-treatment plants (Wagner *et al.*, 1993, 1994b; Manz *et al.*, 1994; Wallner *et al.*, 1995; Snajdr *et al.*, 1997; Wagner & Amann, 1997; Wagner & Loy, 2002). Several filamentous β -*Proteobacteria* contribute to bulking problems of activated sludge (Wagner *et al.*, 1994a) whereas other β -*Proteobacteria* catalyse, for example, aerobic ammonia oxidation (Purkhold *et al.*, 2000; Juretschko *et al.*, 2002), denitrification (Ginige *et al.*, 2004) or the removal of phosphorous from sewage (Lee *et al.*, 1999, 2003; Hesselmann *et al.*, 1999; Blackall *et al.*, 2002). However, cultivation-independent methods that are based solely on phylogenetic sequence information are not adequate to reveal an encompassing picture of the physiology of these

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Abbreviations: EBPR, enhanced biological phosphorus removal; PAO, phosphate-accumulating organism; PHA, polyhydroxyalkanoate; poly-P, polyphosphate.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain P1^T is AJ627188.

Additional micrographs and an unrooted phylogenetic tree are available as supplementary material in IJSEM Online.

micro-organisms in the environment. In particular, bacteria belonging to the family *Comamonadaceae* and neighbouring phylogenetic groups are phenotypically highly diverse, even if they are phylogenetically closely related, such that it is very difficult to correlate a distinct phylotype found in the environment with a certain metabolic potential of a related recognized species.

Two approaches have been developed to overcome these limitations in studying the physiological potential of distinct bacterial phylotypes in sewage sludge. Lee *et al.* (1999) and Ouverney & Fuhrman (1999) introduced a novel tool in microbial ecology by combining the oligonucleotide-based fluorescence *in situ* hybridization (FISH) of single cells with a high-resolution measurement of the microbial substrate uptake pattern using microautoradiography. A less laborious approach is based on screening of novel isolates retrieved from the studied environment with oligonucleotide probes targeting a specific group of micro-organisms. Pure cultures of the strains obtained can then be studied *ex situ* by applying standard microbial methods. An oligonucleotide-probe-assisted directed cultivation procedure has been successfully applied to samples of activated sludge and led to the characterization of several novel species (Schulze *et al.*, 1999; Spring *et al.*, 2004; Kämpfer *et al.*, 2005).

The oligonucleotide probe LDI23a (Wagner *et al.*, 1994a), specific for *Leptothrix discophora* and several related species within the *Comamonadaceae*, was used for the targeted isolation of novel β -*Proteobacteria* from activated sludge of a large municipal wastewater treatment plant (München I; Großlappen, Germany). Probe LDI23a was chosen because in the high-load aeration basin of this plant abundant bacteria hybridizing with this probe *in situ* were observed by FISH. Screening of isolates using this probe identified two novel strains. The complete isolation procedure and characterization of one of these strains have been described previously by Spring *et al.* (2004). A poly-phasic characterization of the second strain, designated P1^T, is presented here.

Standard microbial methods, as reported previously by Spring *et al.* (2001, 2004), were used for the phenotypic characterization of the newly isolated strain P1^T and of *Pseudomonas spinosa* ATCC 14606^T, obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). *P. spinosa* was chosen for a comparative characterization because it represents the phylogenetically most closely related species and the description provided by Leifson (1962) is rather sparse. Unless stated otherwise, R2A medium (Reasoner & Geldreich, 1985) was used for growth experiments. Cells were Gram-negative, straight to slightly curved rods with rounded ends (Fig. 1a). They occurred singly or in short chains and were motile by means of one to two polar flagella. Filamentous cells up to 50 μ m in length were frequently observed in nutrient-rich media. Under conditions of nitrogen limitation highly refractile intracellular granules could be observed

Fig. 1. Phase-contrast micrographs of strain P1^T demonstrating the accumulation of PHA in the form of refractile granules under conditions of nitrogen limitation. (a) Growth in modified SFW medium containing 2.0 g acetate l⁻¹ and 0.4 g NH₄Cl l⁻¹; (b) growth in modified SFW medium containing 2.0 g acetate l⁻¹ without added NH₄Cl. Bar, 10 μ m.

by phase-contrast microscopy. After prolonged incubation in media that contained only very low concentrations of bound nitrogen, cells appeared slightly swollen and were almost completely filled with these granules (Fig. 1b). Storage inclusions consisted of polyhydroxyalkanoates (PHAs), which could be demonstrated by staining with Nile Blue (Ostle & Holt, 1982). An epifluorescence micrograph demonstrating the typical bright red–orange fluorescence of the PHA granules after staining with Nile Blue is available as supplementary Fig. A in IJSEM Online.

Storage of polyphosphate (poly-P) as reserve material in cells of strain P1^T was shown by Neisser staining according to the protocol of Jenkins *et al.* (1993). Both kinds of reserve polymers could also be detected in the closely related strain *P. spinosa* ATCC 14606^T.

Colonies of strain P1^T appeared after 24 h on R2A agar. They reached about 2 mm in diameter after 2 days incubation. After several days colonies appeared mucoid as a result of the formation of extracellular slime. No diffusible pigments were formed.

The newly isolated strain grew well at temperatures ranging from 5 to 40 °C (temperatures below 5 °C were not tested). The pH range for growth in buffered R2A broth was 6.0–9.0. Adaptation of strain P1^T to freshwater habitats is reflected in its low salt tolerance. NaCl is tolerated in the medium only up to a concentration of 0.5 % (w/v). Under optimal growth conditions (35 °C, pH 7.0) the doubling time of P1^T was 1.2 h. The growth characteristics of *P. spinosa* ATCC 14606^T were similar to P1^T with only marginal differences; these are explicitly given in the formal species descriptions below.

The spectrum of usable carbon sources was determined in a microplate assay as described by Kämpfer *et al.* (1991) with the modification that the final substrate concentrations were 0.1 % (w/v). Strain P1^T was able to utilize a broad range of different substrates as sole carbon sources, including carbohydrates, sugar alcohols and organic acids. Formation of PHA granules was not restricted to a specific substrate, but acetate was particularly suitable to demonstrate its accumulation in this strain. In contrast, the substrate utilization pattern of *P. spinosa* ATCC 14606^T was more limited and restricted to a few carbohydrates and organic acids.

Chemolithoautotrophic growth of the novel strain P1^T and *P. spinosa* ATCC 14606^T with hydrogen as substrate was tested under the conditions described by Malik & Schlegel (1981) for the cultivation of various Knallgas bacteria. As a trait that distinguishes both strains from most species of the closely related genus *Hydrogenophaga*, no aerobic autotrophic growth with hydrogen as substrate was detected. Thiosulfate was not utilized as substrate and did not support autotrophic or chemolithoheterotrophic growth. In contrast, two species of the genus *Hydrogenophaga*, *Hydrogenophaga palleronii* and *Hydrogenophaga intermedia*, were shown to oxidize thiosulfate to sulfate (Kämpfer *et al.*, 2005).

Strains P1^T and *P. spinosa* ATCC 14606^T were both oligonitrophilic and could grow in liquid mineral medium with 0.1 g yeast extract l⁻¹ as the only source of bound nitrogen. Fixing of molecular nitrogen, however, could not be demonstrated. No growth was observed under anaerobic conditions. Nitrate was reduced to nitrite, but denitrification did not occur. Neither strain was able to grow phototrophically.

Further details of the morphological and physiological characterization of strain P1^T and *P. spinosa* ATCC 14606^T are given in Table 1 and in the formal species descriptions below.

Analysis of the respiratory lipoquinones of strains P1^T and *P. spinosa* ATCC 14606^T was by HPLC and electron-impact mass spectrometry according to the methods of Monciardini *et al.* (2003); ubiquinone-8 (Q-8) was the predominant component in both strains. This quinone system is a characteristic feature of the β -Proteobacteria

(Yokota *et al.*, 1992). The cellular fatty acid composition of both strains was analysed after growth on R2A agar and TSA plates (Difco) and compared with the fatty acid pattern of *Macromonas bipunctata* DSM 12705^T, the only available pure culture of the genus *Macromonas* (Table 2). Fatty acid methyl esters were extracted and prepared by the standard protocol of the Microbial Identification System (MIDI, Microbial ID). Extracts were analysed by GLC as described by Kämpfer & Kroppenstedt (1996) and Kämpfer *et al.* (1997). The fatty acid profiles of strains P1^T and *P. spinosa* ATCC 14606^T were very similar but distinguishable. In general, the patterns of both strains were typical for members of the *Comamonadaceae* and were dominated by the unbranched fatty acids 16:1 ω 7c (54–71 %), 16:0 (15–25 %) and 18:1 ω 7c (6–12 %). The abundance of distinct fatty acids depended largely on the incubation conditions. For example, in both strains the hydroxylated fatty acid 8:0 3-OH was only detected after incubation on TSA plates. One possible reason for this effect could be the much higher protein content of TSA medium compared to R2A agar. Significant differences were found for the fatty acid profile of *Macromonas bipunctata* DSM 12705^T, which was dominated by unsaturated fatty acids 16:1 ω 7c, 17:1 ω 6c and 18:1 ω 7c, whereas 16:0 was present only in minor amounts.

A comparison of the fatty acid profiles obtained with the overall fatty acid composition of the genus *Hydrogenophaga* is shown in Table 2. The large number of type strains belonging to this genus makes detailed comparison with related taxa consisting of only one or two strains difficult. Nevertheless, the occurrence of the cyclo propane fatty acid 17:0 cyclo among *Hydrogenophaga* species could be used to distinguish it from its most closely related taxa.

The 16S rRNA gene of strain P1^T was amplified and sequenced as reported by Spring *et al.* (2001). A continuous stretch of 1525 nt of the 16S rRNA gene sequence of strain P1^T was determined and used for a comparative phylogenetic analysis applying the ARB software package, including a current release of the small-subunit rRNA database (Ludwig *et al.*, 2004). An ARB parsimony analysis placed strain P1^T within the family *Comamonadaceae* of the β -Proteobacteria. In various phylogenetic trees reconstructed by using neighbour-joining, maximum-likelihood or maximum-parsimony methods, the nearest relatives of strain P1^T were *Hydrogenophaga* species, *Macromonas bipunctata* and *P. spinosa*. In most of the reconstructed trees these bacteria formed a coherent lineage supported by bootstrap values of 80 % or greater. A representative phylogenetic tree including bootstrap values is shown in Fig. 2. In this tree, which is based on only a limited number of sequences representing mainly type species of the *Comamonadaceae*, some members of the genus *Hydrogenophaga* form a common branch with strain P1^T, *P. spinosa* and *Macromonas bipunctata*. However, when extensive datasets were used for tree reconstruction that comprise all available, nearly complete 16S rRNA gene sequences

Table 1. Differential characteristics of the genus *Malikia* and closely related genera in the family *Comamonadaceae*

All type strains of the genera listed have a rod-shaped morphology, are oxidase- and catalase-positive, are able to grow at 35 °C, and accumulate PHAs and poly-P as reserve polymers. Symbols: +, present in all type strains; –, absent from all type strains; d, 11–89% of type strains positive; ND, not determined. Data for the genera *Hydrogenophaga* and *Macromonas* were taken from Kämpfer *et al.* (2005) and Dubinina & Grabovich (1984), respectively.

Characteristic	<i>Malikia</i>	<i>Hydrogenophaga</i>	<i>Macromonas</i>
No. of species (<i>n</i>)	2	7	2*
Cell diameter (µm)	1.1–1.3	0.3–0.6	2.2–4.5
Flagella	1–3, polar	1, polar	Polar tuft
PHA accumulation induced by N limitation	+	+	–
Non-diffusible pigments	–	+	–
Slime formation on R2A agar	+	–	–
Autotrophic growth with H ₂	–	d	–
Reduction of NO ₃ [–]	+	d	–
Reduction of NO ₂ [–]	–	d	–
Oxidation of S ₂ O ₃ ^{2–}	–	d	–†
Growth factors required	+	–	+
Utilization of:			
D-Fructose	–	d	–
D-Glucose	+	d	–
Formate	–	d	+
Acetate	d	–	+
Fumarate	d	d	+
DL-Lactate	d	+	+
Glycerol	–	+	–
D-Mannitol	–	d	ND
Major 3-OH fatty acids	8:0	8:0 or 10:0	8:0
DNA G + C content (mol%)	66–67	64–69	68

*Data for this genus are based on the type strain of the only available species, *Macromonas bipunctata*. The type species of the genus, *Macromonas mobilis*, is not available in pure culture.

†Thiosulfate oxidation by cells of *Macromonas bipunctata* is due to chemical oxidation by H₂O₂ (Dubinina & Grabovich, 1984).

affiliated to the *Comamonadaceae*, strain P1^T was located on a common lineage together with *P. spinosa* and *Macromonas bipunctata*, but separated from *Hydrogenophaga* species (this tree is available as supplementary Fig. B in IJSEM Online). The phylogenetically most closely related species to strain P1^T, based on 16S rRNA gene sequence similarity values, was *P. spinosa* (98.4%). This species can be regarded as misclassified, because it is phylogenetically only distantly related to *Pseudomonas aeruginosa*, the type species of the genus. 16S rRNA gene sequence similarity values between P1^T and the other closely related taxa that form a common phylogenetic branch in Fig. 2 ranged from 95.3 to 96.9%.

Genomic DNA for the determination of DNA base composition and DNA–DNA hybridization experiments was isolated using a French pressure cell and purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). The G + C content of the total DNA of strain

P1^T and *P. spinosa* ATCC 14606^T was 67 and 66 mol%, respectively. DNA–DNA hybridization experiments between both strains were carried out in 2 × SSC buffer containing 10% DMSO at 70 °C according to the method of De Ley *et al.* (1970). DNA renaturation rates were measured using a Gilford System 2600 spectrophotometer (Gilford Instrument Laboratories) equipped with a Gilford 2527-R thermoprogrammer and plotter. The estimated level of DNA–DNA binding between strains P1^T and *P. spinosa* ATCC 14606^T was 53%, indicating that both strains represent distinct species.

A peculiarity of the novel strain P1^T and *P. spinosa* ATCC 14606^T was the intracellular accumulation of PHAs and poly-P. Micro-organisms that are able to store these compounds in large amounts are thought to play a major role in the enhanced biological phosphorus removal (EBPR) process of specially designed wastewater treatment plants, which treat the sewage by alternating exposure to anaerobic

Table 2. Fatty acid pattern of the novel strain P1^T compared to patterns of *P. spinosa* ATCC 14606^T, *Macromonas bipunctata* DSM 12705^T and representatives of the genus *Hydrogenophaga*

Strains P1^T and *P. spinosa* ATCC 14606^T were incubated on either R2A agar or TSA plates (Difco) at 28 °C for 24 h, whereas *Macromonas bipunctata* DSM 12705^T was incubated only on R2A agar for 7 days prior to fatty acid analysis. The *Hydrogenophaga* type strains were incubated on TSA plates at 28 °C for 48 h. Values are percentages of total fatty acids. Gaps indicate that the fatty acid was not detected. The three major fatty acids are given in bold type. The position of the double bond in unsaturated fatty acids is located by counting from the methyl (ω) end of the carbon chain; *cis* and *trans* isomers are indicated by the suffixes *c* and *t*, respectively. Abbreviations: tr, trace; cyc, cyclopropane; alde, aldehyde.

Fatty acid	P1 ^T		<i>P. spinosa</i>		<i>Macromonas bipunctata</i>	<i>Hydrogenophaga</i>
	R2A	TSA	R2A	TSA	R2A	TSA
Saturated fatty acids						
9:0					0.2	0–0.1
10:0				0.1		0–tr
11:0						0–0.9
12:0						0–6.0
13:0						0–1.7
14:0	1.4	1.2	1.1	1.7	2.4	0–3.6
15:0		3.2			1.2	0–4.9
16:0	14.7	19.9	25.0	14.8	5.3	2.3–42.9
17:0		0.9			0.8	0.2–6.6
18:0						0–0.7
Unsaturated fatty acids						
15:1 ω 6 <i>c</i>					0.9	0–14.8
16:1 ω 5 <i>c</i>	3.2	1.3	1.7	2.7	1.1	0–0.6
16:1 ω 7 <i>c</i>	71.0	53.9	57.9	71.1	62.5	16.2–51.1
17:1 ω 6 <i>c</i>		3.8			12.5	0–20.4
17:1 ω 8 <i>c</i>		1.3			1.1	0–2.6
18:1 ω 7 <i>c</i>	6.3	10.4	12.8	6.9	11.9	9.8–25.6
Hydroxy fatty acids						
8:0 3-OH		2.7		2.8	0.2	0–3.8
9:0 3-OH		0.5				0–1.0
10:0 3-OH						0–3.2
16:0 3-OH						0–0.3
Cyclo propane acids						
17:0 cyc						0–17.2
19:0 cyc ω 8 <i>c</i>						0–1.5
Branched chain fatty acids						
16:0 iso						0–0.7
19:0 iso						0–1.6
Summed features*						
Summed feature 2				tr		
Summed feature 7	2.3	1.1	1.6		0.3	

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 2 contained one or more of the following fatty acids: 12:0 alde, 16:1 iso I and/or 14:0 3-OH. Summed feature 7 contained one or more of the following isomers: 19:1 ω 6*c*, 19:0 cyc and/or an unknown compound with the equivalent chain-length 18:846.

and aerobic conditions (Blackall *et al.*, 2002; Serafim *et al.*, 2002). According to current models of this process (Seviour *et al.*, 2003) extracellular phosphorus is removed from the wastewater by phosphate-accumulating organisms (PAOs) that take up phosphorus under aerobic conditions and

store it as poly-P. Under anaerobic conditions the accumulated poly-P is degraded to provide energy for the PAOs to assimilate organic substances and to synthesize PHAs, which again serve under aerobic conditions (in the absence of extracellular organic compounds) as carbon and energy

Fig. 2. Phylogenetic dendrogram illustrating the position of the newly proposed genus *Malikia* among representatives of the Comamonadaceae. With the exception of the genera *Macromonas* and *Hydrogenophaga*, only 16S rRNA gene sequences of recognized type species within the Comamonadaceae were used for tree reconstruction. GenBank/EMBL/DDBJ accession numbers are given in parentheses. The tree was derived from a distance matrix using the method of Saitou & Nei (1987) as implemented in the ARB (PHYLIP) package (Ludwig *et al.*, 2004). Phylogenetic distances were calculated as described by Felsenstein (1982). Only bootstrap values above 70% (1000 bootstrap resamplings for each node) are shown. The sequence of *Escherichia coli* (GenBank/EMBL/DDBJ accession no. L10328) was used as an outgroup (not shown). Bar, estimated sequence divergence.

source. In addition, the reserve polymer glycogen is probably used as a source for reducing equivalents under anaerobic conditions. Hence, the cycling of reserve polymers provides a selective advantage of PAOs compared to other bacteria. It is assumed that the anaerobic–aerobic cycling of biomass during the EBPR process leads to selective enrichment of PAOs under the feast–famine regime in EBPR plants (Serafim *et al.*, 2002). Use of intracellular polymers via the process described above is probably more efficient and faster as a complete metabolic switch from aerobiosis to anaerobiosis, as observed in many facultatively anaerobic, denitrifying bacteria isolated from wastewater.

The accumulation of poly-P and PHAs in strain P1^T was studied under various conditions in order to reveal the possible mechanisms that regulate synthesis of reserve polymers in this strain. The results were compared to the accumulation of reserve polymers in phylogenetically related bacteria. Although it is difficult to identify true PAOs involved in the EBPR process based solely on *ex situ* experiments, the results obtained should provide valuable taxonomic information regarding the internal metabolism in strain P1^T compared to related bacteria.

In these experiments a simple freshwater medium based on the SFW medium of Spring *et al.* (2004) was used. The modified SFW medium contained, per litre of distilled water: 0.8 g K₂HPO₄, 0.3 g KH₂PO₄, 0.4 g MgSO₄·7H₂O, 2.0 ml trace elements solution (Vishniac & Santer, 1957),

1.0 ml selenite/tungstate solution (Tschech & Pfennig, 1984), 0.1 g yeast extract (Difco) and 10.0 ml vitamins solution (Wolin *et al.*, 1963), to a final pH of 7.2. Substrates and a source of bound nitrogen (NH₄Cl) were added after autoclaving at the concentrations indicated below. Anoxic media were prepared by applying the anaerobe culture technique of Hungate (1950) with the modifications introduced by Bryant (1972).

No growth of strain P1^T and *P. spinosa* ATCC 14606^T was observed on substrates in modified SFW medium lacking vitamins or yeast extract, indicating that both strains cannot proliferate without growth factors. To study the effect of nitrogen limitation on the accumulation of reserve polymers in strain P1^T, the modified SFW medium was supplemented with 2.0 g sodium acetate l⁻¹, but not with NH₄Cl. Hence, the only source of bound nitrogen in this medium was 0.1 g yeast extract l⁻¹. A loopful of P1^T cells grown on R2A agar plates was transferred to this medium and incubated for 24 h. Cells incubated under nitrogen limitation were viewed by phase-contrast microscopy to detect visible inclusions and were tested for poly-P accumulation with Neisser staining. If visible inclusions were observed, the presence of PHA was subsequently confirmed by staining with Nile Blue. In P1^T cells grown on R2A agar, generally only low to moderate amounts of poly-P and PHA could be detected by the respective staining procedures. In contrast, incubation under nitrogen limitation resulted in the accumulation of large amounts

of PHA in the form of highly refractile granules, whereas poly-P was no longer detectable within the cells. A photomicrograph illustrating the absence of poly-P in nitrogen-starved cells by Neisser staining is available as supplementary Fig. C(a) in IJSEM Online. Interestingly, no accumulation of PHAs was observed in the same medium under anoxic or micro-oxic conditions (gas atmosphere containing 1 % oxygen).

In a complementary experiment, cells of strain P1^T were incubated in modified SFW medium containing 0.4 g NH₄Cl l⁻¹ as nitrogen source, but no acetate. After 24 h incubation, intracellular PHAs were almost completely degraded and large amounts of intracellular poly-P had accumulated. A photomicrograph illustrating the storage of poly-P in carbon-starved cells by Neisser staining is available as supplementary Fig. C(b) in IJSEM Online.

The above experiments imply that the accumulation of reserve compounds in this strain depends largely on the lack of nitrogen or carbon in the medium. However, to determine if this strain could represent a typical PAO, more laborious analyses would be necessary following incubations under alternate anaerobic/oxic conditions, for example as described by Stante *et al.* (1997).

Similar results were obtained with *P. spinosa* ATCC 14606^T. The only difference was that acetate could not be utilized for the production of PHAs. However, acetate could be replaced with the substrate maltose, which was also effective in inducing the production of PHAs under nitrogen limitation.

In similar experiments with all known type strains of the genus *Hydrogenophaga*, the same general conditions were effective among species of this genus in the induction of reserve polymers as in strains P1^T and *P. spinosa* ATCC 14606^T. However, the results were not always clear-cut; several strains showed only weak growth under conditions of nitrogen limitation and PHA accumulation depended strongly on the carbon source used. In most strains a significant accumulation of PHAs was only observed with 3-hydroxybutyrate as substrate.

In *Macromonas bipunctata*, although closely related with the above-mentioned species, the production of intracellular reserve compounds seemed to be regulated in a different way. In this species, nitrogen limitation obviously had no effect on the production of PHAs. In contrast, the accumulation of PHAs in *Macromonas bipunctata* DSM 12705^T was dependent upon the carbon source, with acetate being most effective (see also Dubinina & Grabovich, 1984). Under aerobic conditions storage of poly-P was almost constitutive instead of being induced by the deprivation of nutrients. In a preliminary experiment it was shown that a shift from aerobic to anaerobic incubation conditions led to reduced amounts of intracellular poly-P. Although these experiments were only performed with cultures grown on R2A agar plates and the amount of intracellular poly-P was

only estimated by visual inspection after Neisser staining, the results obtained may indicate that poly-P could be utilized by *Macromonas bipunctata* DSM 12705^T under conditions of low oxygen concentrations. Regardless, the internal metabolism of reserve polymers in *Macromonas bipunctata* DSM 12705^T is clearly different from the metabolic type found in the closely related strains P1^T and *P. spinosa* ATCC 14606^T and hence represents a trait of taxonomic relevance.

From the results detailed above, it can be concluded that strain P1^T represents a novel taxon within the family *Comamonadaceae*, for which we propose the name *Malikia granosa* gen. nov., sp. nov. The proposal of a new genus seems justified based on the results of a polyphasic approach including biochemical, physiological and genotypic methods. It is further proposed to transfer the misclassified species *Pseudomonas spinosa* to *Malikia* gen. nov., as *Malikia spinosa* comb. nov. Characteristics that differentiate the new genus *Malikia* from the most closely related taxa of the *Comamonadaceae* are listed in Table 1.

Description of *Malikia* gen. nov.

Malikia (Ma.li'ki.a. N.L. fem. n. *Malikia* named after Kuhrsheed A. Malik, for his contributions to our knowledge of the cultivation and taxonomy of hydrogen-oxidizing and polyhydroxyalkanoate-accumulating proteobacteria).

Gram-negative, rod-shaped cells, motile by polar flagella. PHAs and poly-Ps are stored intracellularly as reserve materials. Endospores or cysts are not formed. Strictly aerobic; catalase- and oxidase-positive. Not able to grow autotrophically or phototrophically. Not able to fix molecular nitrogen. Growth factors are required. Mesophilic. Restricted to freshwater habitats. No growth above 1 % (w/v) NaCl. Major respiratory lipoquinone is ubiquinone 8 (Q-8). Major cellular fatty acids are 16:1 ω 7c, 16:0 and 18:1 ω 7c. The only detectable hydroxylated fatty acid is 8:0 3-OH. Cyclopropane-substituted fatty acids are not present. Phylogenetically affiliated to the *Comamonadaceae* within the β -*Proteobacteria*. The DNA G+C content of the type species is 67 mol% (HPLC).

The type species is *Malikia granosa*.

Description of *Malikia granosa* sp. nov.

Malikia granosa (gra.no'sa. L. fem. adj. *granosa* granular).

Cells are straight to slightly curved rods with rounded ends, 1.3 μ m wide and 3.5–6 μ m long, occurring singly, in pairs or in short chains. Occasionally filaments up to 50 μ m long are formed. Motile by one to two polar flagella. Under conditions of nitrogen limitation cells accumulate large amounts of PHAs that are visible as highly refractile granules by phase-contrast microscopy. Poly-Ps are accumulated under conditions of carbon starvation. Colonies appear after 24 h on R2A agar and reach approximately 2 mm in diameter after 2 days incubation.

They are smooth, circular, raised, cream-white and translucent. After several days colonies appear mucoid owing to the formation of extracellular slime. No diffusible pigments are formed. Growth in liquid medium is homogeneous without formation of visible aggregates. Temperature and pH optima for growth are 35 °C and 6.5–7.0, respectively. The upper temperature limit for growth is about 40 °C. Growth occurs in media containing 0.5 % (w/v) NaCl but not 1 % (w/v) NaCl. Substrates utilized for growth: acetate, adipate, benzoate, gluconate, DL-3-hydroxybutyrate, DL-lactate, L-malate, propionate, pyruvate, D-mannitol, D-glucose, D-mannose, sucrose and L-proline. Substrates not utilized for growth: citrate, fumarate, glycolate, glyoxylate, malonate, oxaloacetate, 2-oxoglutarate, succinate, adonitol, glycerol, arabinose, D-galactose, D-fructose, D-maltose, lactose, L-glutamate and L-histidine. Nitrate is reduced to nitrite. Hydrogen cannot be utilized for chemolithoautotrophic growth. Thiosulfate is not oxidized. The DNA G + C content is 67 mol% (HPLC).

The type strain, P1^T (= DSM 15619^T = JCM 12706^T = CIP 108194^T), was isolated from activated sludge of a high-load basin of a municipal wastewater treatment plant in Munich, Germany.

Description of *Malikia spinosa* comb. nov.

Malikia spinosa (spi.no'sa. L. adj. *spinosa* thorny, spiny).

Basonym: *Pseudomonas spinosa* Leifson 1962 (Approved Lists 1980).

The results of this study confirm to a large extent the data provided by Leifson (1962) in the original description. However, additional data obtained in this study make an emendation of the species description necessary. Cells are straight to slightly curved rods with rounded ends, 1.1 µm wide and 3–6 µm long, occurring singly, in pairs or in short chains. Occasionally filaments up to 50 µm long are formed. Motile by one to three polar flagella. Under conditions of nitrogen limitation cells accumulate large amounts of PHAs that are visible as highly refractile granules by phase-contrast microscopy. Poly-Ps are accumulated under conditions of carbon starvation. Colonies appear after 24 h on R2A agar and reach approximately 1 mm in diameter after 2 days incubation. They are smooth, circular, raised, cream-white and translucent. After several days colonies appear mucoid owing to the formation of extracellular slime. No diffusible pigments are formed. Growth in liquid medium is homogeneous without formation of visible aggregates. Temperature and pH optima for growth are 32 °C and 7.5–8.0, respectively. The upper temperature limit for growth is about 36 °C. Growth in media containing 1 % (w/v) NaCl is weak; no growth occurs above 1 % (w/v) NaCl. Substrates utilized for growth: adipate, gluconate, fumarate, L-malate, D-mannitol, D-glucose, D-maltose and sucrose. Substrates not utilized for growth: acetate, benzoate, citrate, glycolate, glyoxylate, DL-lactate, malonate,

propionate, pyruvate, oxaloacetate, 2-oxoglutarate, succinate, adonitol, glycerol, arabinose, D-galactose, D-fructose, D-mannose, lactose, L-glutamate, L-histidine and L-proline. Nitrate is reduced to nitrite. Hydrogen cannot be utilized for chemolithoautotrophic growth. Thiosulfate is not oxidized. The DNA G + C content is 66 mol% (HPLC).

Isolated from river water (DuPage River, IL, USA). The type strain is ATCC 14606^T (= DSM 15801^T).

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