Catellibacterium nectariphilum gen. nov., sp. nov., which requires a diffusible compound from a strain related to the genus *Sphingomonas* for vigorous growth

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A bacterial strain, designated AST4^T, was isolated from activated sludge. The bacterium did not show significant growth on nutrient broth, but growth was clearly stimulated by addition of supernatant from other bacterial cultures. Culture filtrate of a strain related to the genus *Sphingomonas* in particular increased the cell yield and growth rate of strain AST4^T. Phylogenetic analysis based on the 16S rRNA gene sequences showed that strain AST4^T is located within the '*Rhodobacter* group' in the α -3 subclass of *Proteobacteria*, but is clearly distant from related genera in this group such as *Paracoccus*, *Rhodobacter* and *Rhodovulum*. Strain AST4^T is a Gram-negative, non-motile, rod-shaped (0.6–0.8 × 1.3–2.0 µm) and aerobic bacterium. It was not able to reduce nitrate to nitrite or N₂. No phototrophic growth was observed. Optimal growth occurred at 30 °C and pH 6.5–7.5. The dominant cellular fatty acid in the isolate was C_{18:1} cis11. Ubiquinone-10 was the major respiratory quinone. The G + C content was 64.5 mol% (by HPLC). Based on the phylogenetic and phenotypic traits, the name *Catellibacterium nectariphilum* gen. nov., sp. nov. is proposed for this isolate; the type strain is AST4^T (=NBRC 100046^T = JCM 11959^T = DSM 15620^T).

The majority of micro-organisms distributed in nature are not cultivable by conventional techniques (Amann *et al.*, 1995). One of the primary reasons for such uncultivability is our lack of knowledge about syntrophic relationships between micro-organisms, since some bacterial strains that can not grow on artificial media alone have been cultured in the presence of other bacteria (Ohno *et al.*, 2000; Rhee *et al.*,

2000; Kaeberlein *et al.*, 2002). To obtain such bacteria, they must be screened with growth factors supplied by the other bacterium. In the course of such an attempt, we isolated a bacterial strain that required a diffusible compound from other bacteria for vigorous growth from an activated sludge. The isolate, designated strain AST4^T, gave weak growth on a nutrient broth, but the growth rate and cell yield were stimulated specifically by the addition of supernatant from a strain related to the genus *Sphingomonas*. Strain AST4^T was phylogenetically distant from known bacteria and showed several properties that distinguished it from its other relatives. On the basis of phenotypic and phylogenetic data, we propose that strain AST4^T belongs to a new genus and species, *Catellibacterium nectariphilum* gen. nov., sp. nov. The characteristics of this bacterium are described herein.

The novel isolate was obtained by the following procedure. An activated sludge sample (50 µg) collected from the Kawasaki plant of Ajinomoto Co. (Japan) was added to 100 ml NPB medium (pH 7.0) (containing 10.0 g tryptone

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains AST4^T and GF9 are AB101543 and AB101552, respectively.

An extended 16S rDNA-based neighbour-joining tree containing all species in the genera *Paracoccus*, *Rhodobacter* and *Rhodovulum* is available as supplementary material in IJSEM Online.

peptone, 2.0 g yeast extract, 1.0 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g K_2HPO_4 , 0.5 g KH_2PO_4 and 5.0 g D-glucose in 1 l distilled water) and incubated for 5 days at 30 °C. The resultant culture, which was designated 'bacterial mixed culture', was centrifuged at 15 000 r.p.m. for 10 min and the autoclaved (121 °C, 20 min) supernatant was added to NPB medium at a final concentration of 10 %. This medium was named NPBC (NPB containing mixed culture extract) and used as isolation medium for strains requiring growth factors from other bacteria. An aliquot of activated sludge sample diluted to 10^{-4} with sterilized water was inoculated on a 1.5 % agar plate. After incubation at 30 °C for 4 days, colonies that emerged on the agar plates were picked and isolated. The requirement for a bacterial growth factor (or stimulator) supplied by other bacteria by each isolate was verified by comparing growth between the NPBC agar plate (with the supernatant of bacterial mixed culture) and NPB agar plate (without the supernatant). Consequently, we obtained 30 isolates. Almost all strains did not show significant differences between growth on NPBC and NPB agar plates. However, one strain, designated strain AST4^T, showed a clear difference. Although this strain showed weak growth in NPB medium, addition of the supernatant from the bacterial mixed culture markedly increased the growth rate and cell yield (Table 1).

We also tried to refine the NPBC medium, since it contained the supernatant of bacterial mixed culture, an undefined additive. We searched within the bacterial mixed culture for organisms that could stimulate the growth of strain AST4^T alone, and isolated a novel strain, designated GF9. Supernatants prepared from NPB medium culture of strain GF9 greatly elevated the growth yield of strain AST4^T as well as that of 'bacterial mixed cultures'. The 16S rRNA gene of strain GF9 was partially sequenced (corresponding to positions 912–1389 of *Escherichia coli* 16S rRNA gene), and the sequence obtained showed 99.8 and 99.2 % similarity to those of *Sphingomonas adhaesiva* (Takeuchi *et al.*, 1994) and *Sphingopyxis terrae* (Takeuchi *et al.*, 1994). Based on phylogenetic comparison, the strain was considered to be a member of the genus *Sphingomonas* (*Sphingomonas* sp. strain GF9). In all cultures of AST4^T for the following tests and analyses, NPB medium supplemented with 10 %

supernatant from stationary-phase *Sphingomonas* sp. strain GF9 (NPBGF9 medium) was used.

The morphological, physiological and phylogenetic characteristics of strain AST4^T were investigated. The strain formed smooth, circular, white to beige colonies on 1.5 % agar plates after 36–48 h incubation at 30 °C. The size, shape and ultrastructure of the cells were examined by phase-contrast microscopy and transmission electron microscopy (Fig. 1). Cells of strain AST4^T were non-motile, Gram-negative and ovoid to rod-shaped, 1.3–2.0 µm long and 0.6–0.8 µm wide. Formation of pairs or chains of cells was

Fig. 1. Phase-contrast photomicrograph (a; bar, 10 µm) and electron micrograph of ultrathin section (b; bar, 0.5 µm) of cells of strain AST4^T. Cells were grown on NPBGF9 medium at 30 °C for 24 h. For transmission electron microscopy, a high-pressure freezing method (Yamaguchi *et al.*, 2002) was used. After freeze-substitution, ultrathin sections of the sample were prepared as described previously (Hanada *et al.*, 2002). Samples were stained with uranyl acetate and lead citrate and examined with a Hitachi H-7000 transmission electron microscope.

Table 1. Growth of strain AST4^T in the NPB and NPBC medium

Cells were grown in 5 ml of each medium at 30 °C with shaking (160 r.p.m.). Growth was measured as OD₆₆₀. Standard deviations are shown in parentheses (*n* = 3).

Medium	Growth of strain AST4 ^T			
	1 day	2 days	3 days	4 days
NPB	0.05 (0.01)	0.13 (0.01)	0.25 (0.01)	0.37 (0.01)
NPBC	0.29 (0.03)	0.91 (0.02)	1.01 (0.02)	0.98 (0.01)

frequently observed. Since cells tended to remain attached to the chains, cell aggregates were usually formed in liquid medium. An electron micrograph of a thin section revealed that the isolate has the typical Gram-negative cell-wall structure with an outer membrane and very thin murein layer. Cells did not contain any type of intracytoplasmic membrane. Spherical accumulations of intracellular material were frequently observed and the material was identified as polyhydroxybutyrate by a method using Sudan black B (Jenkins *et al.*, 1993). These storage granules (about 0.3 µm in diameter) were also observed in electron micrographs (Fig. 1b). Fibrous structures on the surface of cell, which would be extracellular polysaccharides, were also seen.

Growth of the strain was investigated in liquid cultures and on 1.5% agar plates. Visible colonies were detectable at 20–37 °C, with optimum growth at 30 °C on NPBGF9 agar. No growth occurred at 15 or 40 °C. Strain AST4^T was able to grow in liquid NPBGF9 medium between pH 6.0 and 8.0 with optimum growth at pH 6.5–7.5 (growth was monitored at OD₆₆₀). When 3% (w/v) NaCl was added to the liquid medium, no growth occurred. The metabolism of strain AST4^T was strictly aerobic and it could not grow anaerobically in NPBGF9 medium supplemented with 0.2% KNO₃. Neither phototrophic nor fermentative growth occurred. Cytochrome oxidase activity was detected by the method using oxidase-testing paper (Nissui Seiyaku). Catalase activity was positive, as formation of bubbles in a 3% H₂O₂ solution was observed. Physiological tests by API systems (bioMérieux) revealed that the isolate showed the following activities. Neither indole production from tryptophan nor gelatin hydrolysis was observed; reduction of nitrate to nitrite or N₂ did not occur; arginine dihydrolase, urease and β-glucosidase were negative. To test oxidation of various carbon sources in the absence of supernatant from a strain GF9 culture, Biolog GN2 microplates (GSI Creos) were used. The isolate oxidized glycogen, Tween 80, methyl pyruvate, β-hydroxybutyrate, α-ketoglutarate, DL-lactate, succinate, succinamate, alaninamide, L-glutamate, L-serine and monomethyl succinate as sole carbon and energy sources. Sugars and sugar alcohols such as α-D-glucose, D-fructose, D-galactose, D-mannose, sucrose, D-sorbitol, turanose and xylitol were not significantly oxidized.

Quinones, fatty acids and the G + C content were analysed by the methods described by Hanada *et al.* (2002). Strain AST4^T contained ubiquinone-10 as the main respiratory quinone. The major fatty acid in the isolate was C_{18:1} cis11, which accounted for 91.0% of total cellular fatty acids. C_{17:0} (3.44%), C_{19:0} cyclo11–12 (2.82%), C_{16:0} (0.86%), C_{16:1} cis9 (0.79%), C_{18:0} (0.72%), C_{17:0} cyclo9–10 (0.21%) and C_{15:0} (0.18%) were also detected as minor components. No hydroxy fatty acid was detected. The G + C content of the DNA of strain AST4^T was 64.5 mol% (by HPLC analysis).

An almost complete 16S rRNA gene sequence of the isolate was obtained by PCR using two oligonucleotide primers,

5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-GGTTACCTGTTACGACTT-3' (corresponding to positions 8–27 and 1492–1510 of the *E. coli* 16S rRNA gene; Weisburg *et al.*, 1991). Phylogenetic analysis of the sequence with the neighbour-joining method (Saitou & Nei, 1987; Thompson *et al.*, 1994) placed strain AST4^T within the 'Rhodobacter group' in the α-3 subclass of *Proteobacteria* (Fig. 2; see also the supplementary figure available in IJSEM Online). The isolate was closely related to the genera *Paracoccus*, *Rhodobacter* and *Rhodovulum*. However, strain AST4^T was clearly distant from any species in these three genera, with sequence similarities of less than 94.7%. These low similarities suggest that the strain differs phylogenetically from related genera and that a new genus should be created for the isolate.

Comparative phenotypic properties of strain AST4^T and representative genera belonging to the 'Rhodobacter group' in the α-3 subclass of *Proteobacteria* are summarized in Table 2. The strain shared several important characteristics with members of the 'Rhodobacter group'; for example, their quinone component and major cellular fatty acid composition were almost identical. However, the following conspicuous phenotypic traits distinguish the novel isolate from the other three genera: (i) strain AST4^T was clearly distinguishable from the genera *Rhodobacter* and *Rhodovulum* by the absence of photosynthetic activity (Hansen & Imhoff, 1985; Imhoff, 1989; Hiraishi *et al.*, 1996; Eckersley & Dow, 1980; Hiraishi & Ueda, 1994, 1995; Straub *et al.*, 1999), and the isolate could not synthesize photosynthetic pigments (bacteriochlorophylls) under any growth conditions tested; (ii) the isolate did not show nitrate reduction activity, which is typical of most *Paracoccus* species (13 of 16 members; Kuenen & Robertson, 1989; Urakami *et al.*, 1989, 1990; Ohara *et al.*, 1990; Katayama *et al.*, 1995; Siller *et al.*, 1996; Lipski *et al.*, 1998; Rainey *et al.*, 1999; Doronina *et al.*, 1998, 2002; Pukall *et al.*, 2003) [the only exceptions are *Paracoccus carotinifaciens* (Tsubokura *et al.*, 1999), *Paracoccus marcusii* (Harker *et al.*, 1998) and *Paracoccus*

Fig. 2. Phylogenetic tree of strain AST4^T and related species based on 16S rRNA gene sequences. Bootstrap percentages are indicated at branching points. Bar, 1 substitution in 100 nt. Accession numbers are shown in parentheses. A tree including more reference species is available as supplementary material in IJSEM Online.

Table 2. Phenotypic characteristics of strain AST4^T and related genera

Data for *Paracoccus* (16 species), *Rhodobacter* (5 species) and *Rhodovulum* (6 species) are based on published results. +, Positive; –, negative; v, variable. The quinone system and major fatty acid of strain AST4^T are Q-10 and C_{18:1}, which are identical to those of members of the other genera.

Characteristic	AST4 ^T	<i>Paracoccus</i>	<i>Rhodobacter</i>	<i>Rhodovulum</i>
Cell shape	Ovoid to rods	Cocci to rods	Ovoid to rods	Ovoid to rods
Motility	–	–*	v†	+
Production of carotenoids	–	–‡	+	+
Phototrophic growth	–	–	+	+
Growth in 3% (w/v) NaCl	–	v§	v	+
Nitrate reduction	–	+¶	v#	–

**P. carotinifaciens* and *Paracoccus versutus* are positive. No data available for *Paracoccus alkenifer*.
†*Rhodobacter capsulatus*, *Rhodobacter sphaeroides* and *Rhodobacter azotoformans* are motile. *Rhodobacter veldkampii* and *Rhodobacter blastica* are non-motile.
‡*P. carotinifaciens*, *P. marcusii* and *P. zeaxanthinifaciens* produce large amounts of carotenoids.
§*Paracoccus seriniphilus*, *Paracoccus denitrificans* and *P. zeaxanthinifaciens* are positive. *Paracoccus alcaliphilus*, *Paracoccus aminophilus* and *Paracoccus aminovorans* give weak growth. *Paracoccus solventivorans*, *Paracoccus methylutens*, *Paracoccus kondratievae* and *Paracoccus kocurii* are negative. No data for *P. alkenifer*, *P. carotinifaciens*, *Paracoccus pantotrophus*, *Paracoccus thiocyanatus* or *P. versutus*.
||*Rhodobacter sphaeroides* and *Rhodobacter azotoformans* positive; *Rhodobacter capsulatus* and *Rhodobacter veldkampii* negative. No data for *Rhodobacter blastica*.
¶*P. carotinifaciens*, *P. marcusii* and *P. zeaxanthinifaciens* are negative.
#Variable at the strain level.

zeaxanthinifaciens (Berry *et al.*, 2003)]; (iii) it is easy to distinguish the isolate from the three non-nitrate-respiring *Paracoccus* species because the latter species produce large amounts of carotenoids such as astaxanthin and zeaxanthin, whereas the novel isolate has no ability to synthesize carotenoids.

Based on these phenotypic and phylogenetic comparisons, we conclude that strain AST4^T represents a new genus within the α -3 subclass of the *Proteobacteria*, for which we propose the name *Catellibacterium nectariphilum* gen. nov., sp. nov.

Description of *Catellibacterium* gen. nov.

Catellibacterium (Ca.tel.li.bac.te'ri.um. L. n. *catella* a small chain; N.L. neut. n. *bacterium* from Gr. n. *bakterion* a small rod; N.L. neut. n. *Catellibacterium* a chained small rod).

Gram-negative, strictly aerobic, non-motile, ovoid to rod-shaped cells, growing in pairs and chains. No growth under anaerobic conditions either by fermentation, nitrate reduction or phototrophy. Oxidase and catalase are positive. Indole production from tryptophan, nitrate reduction and gelatin hydrolysis are negative. Arginine dihydrolase, urease and β -glucosidase activities are absent. Intracellular polyhydroxybutyrate accumulation is observed. Growth occurs under mesophilic and neutrophilic conditions. The major cellular fatty acid is C_{18:1}*cis*11. DNA G+C content of the type strain of the type species is 64.5 mol% (by HPLC). Ubiquinone-10 is the major component of the quinone system. 16S rRNA gene sequence analysis places the genus

in the α -3 subclass of *Proteobacteria*. The type species is *Catellibacterium nectariphilum*.

Description of *Catellibacterium nectariphilum* sp. nov.

Catellibacterium nectariphilum (nec.ta'ri.phil.um. L. neut. n. *nectar* nectar; Gr. adj. *philos* loving; N.L. neut. adj. *nectariphilum* loving nectar, referring to the stimulation of growth by excretions of other bacteria).

Basic phenotypic characteristics are the same as those described for the genus. Cells are ovoid to rod-shaped (0.6–0.8 × 1.3–2.0 μ m), occurring in pairs and chains. Colonies are circular and white to beige in colour. The temperature and pH ranges for growth are 20–37 °C and pH 6.0–8.0. Optimum growth occurs at 30 °C and pH 6.5–7.5. Glycogen, Tween 80, methyl pyruvate, β -hydroxybutyrate, α -ketoglutarate, DL-lactate, succinate, succinamate, alaninamide, L-glutamate, L-serine and monomethyl succinate are utilized as sole carbon sources. Diffusible metabolite(s) of other bacteria may be required for vigorous growth.

The type strain is AST4^T (=NBRC 100046^T=JCM 11959^T=DSM 15620^T), which was isolated from an activated sludge sample.

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