Pusillimonas noertemannii gen. nov., sp. nov., a new member of the family *Alcaligenaceae* that degrades substituted salicylates

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The taxonomic position of a *Pseudomonas*-like strain, designated BN9^T, was investigated. This strain had previously been isolated as a 5-aminosalicylate-degrading organism from a 6-aminonaphthalene-2-sulphonate-degrading mixed bacterial culture. Previously, detection of ubiquinone Q-8, a polyamine pattern with putrescine, spermidine and 2-hydroxyputrescine as the major polyamines, and partial 16S rRNA gene sequencing had suggested that strain BN9^T belongs to the 'Betaproteobacteria'. This was supported by sequencing the 16S rRNA gene, which demonstrated 94–96 % sequence similarity to different species of the genera *Achromobacter*, *Alcaligenes* and *Bordetella*, and suggested that strain BN9^T represents a member of the family *Alcaligenaceae*. Different methods for the construction of phylogenetic dendrograms placed the strain separately from all other genera within the *Alcaligenaceae*. Fatty acid analysis demonstrated the presence of high concentrations of C_{19:0} cyclo ω8c. On the basis of low 16S rRNA gene sequence similarity to other members of the *Alcaligenaceae*, fatty acid and polar lipid profiles, and other unique phenotypic properties of strain BN9^T, the creation of a new genus and species with the name *Pusillimonas noertemannii* gen. nov., sp. nov. is proposed. The type strain is BN9^T (= DSM 10065^T = NCIMB 14020^T).

Strain BN9^T had previously been isolated from a 6-aminonaphthalene-2-sulphonate (6A2NS)-degrading mixed bacterial culture that was originally enriched from water from the River Elbe in Germany. It was subsequently demonstrated that the strain mineralized 5-aminosalicylate (5AS), which was formed as an intermediate compound in the metabolism of 6A2NS in the mixed culture (Nörtemann *et al.*, 1986). The metabolism of 5AS by strain BN9^T was analysed in detail and it was shown that the strain converted 5AS by an extraordinary ring-fission dioxygenase to a non-aromatic aminated ring-fission product (Stolz *et al.*, 1992; Stolz & Knackmuss, 1993a). The strain also degraded 3-aminobenzoate via 5AS and 2,3-, 2,4- and

2,5-dihydroxybenzoate via different degradative pathways (Russ *et al.*, 1994; Stolz, 1989; Stolz & Knackmuss, 1993b).

The organism was originally tentatively identified as a pseudomonad, but an analysis of its quinone system (Q-8), polyamine pattern (mainly putrescine, spermidine and 2-hydroxyputrescine) and a 158 bp sequence of its 16S rRNA suggested that the strain was closely related to the genus *Alcaligenes* (Busse *et al.*, 1992). Here we report a detailed characterization of strain BN9^T.

Strain BN9^T showed only weak growth on all complex [Luria–Bertani (LB) and nutrient broth (NB)] or mineral media tested. Generally, the best growth was observed on chocolate blood agar (Columbia agar base; Oxoid CM331, prepared as recommended by the manufacturer, after cooling to 45 °C, supplemented with 10 % defibrinated sheep blood and boiled) after 3–4 days incubation at 37 °C, which resulted in a colony diameter of approximately 2–3 mm. Growth was slightly slower on blood agar (Oxoid 331, prepared as described above but supplemented with 5 % defibrinated sheep blood and without boiling) under

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Pusillimonas noertemannii* BN9^T is AY695828.

Two-dimensional chromatograph of polar lipids of different members of the *Alcaligenaceae* is available as supplementary material in IJSEM Online.

the same conditions. On complex media such as NB or peptone yeast extract (PYE; 0.3% yeast extract, 0.3% peptone, 1.5% agar, pH 7.2) the colonies reached a diameter of about 1–2 mm within 5–6 days at 30 °C. The colonies were brownish, circular with an entire margin. Growth at 37 °C was slightly faster than at 30 °C. In the presence of increased CO₂ concentrations (5%) no visible differences compared with growth at normal O₂ and CO₂ pressures were observed (at 37 °C). Other growth characteristics are given in the species description below.

The 16S rRNA gene sequence of strain BN9^T, containing a continuous sequence of 1524 bp (positions 46–1502 according to the *Escherichia coli* numbering scheme; Brosius *et al.*, 1978), was used to search the GenBank library. The results of the sequence comparisons demonstrated that strain BN9^T was indeed most closely related to different members within the *Alcaligenaceae*. Phylogenetic trees were generated by using the maximum-parsimony, maximum-likelihood and neighbour-joining methods from the PHYLIP program package (Felsenstein, 1993). In these trees, strain BN9^T grouped separately from the clades formed by the genera *Achromobacter*, *Alcaligenes*, *Bordetella* and *Kerstersia*. A closer relationship was suggested by the sequence comparison programs between strain BN9^T and members of the genera *Taylorella*, *Pelistega* and *Advenella* (Fig. 1). Furthermore, all phylogenetic trees suggested that *Alcaligenes faecalis* DSM 30030^T and *Alcaligenes defragrans* DSM 12141^T (Foss *et al.*, 1998) do not form a coherent group within the clade and therefore presumably do not belong to the same genus.

The fatty acid profile of strain BN9^T showed many of the characteristics also found in species of the genera *Alcaligenes*, *Achromobacter*, *Bordetella*, *Pigmentiphaga*, *Kerstersia*, *Oligella*, *Taylorella* and *Pelistega* (Table 1; Vandamme *et al.*, 1996; Busse & Stolz, 2004). However, the presence of the major compound C_{19:0} cyclo ω 8c was specific for strain BN9^T (Table 1; Busse & Stolz, 2004).

The polar lipid compositions of BN9^T, *Alcaligenes faecalis* DSM 30030^T, *Achromobacter xylosoxidans* KM534^T, *Achromobacter denitrificans* DSM 30026^T and *Alcaligenes defragrans* DSM 12141^T are shown in Table 2 and Fig. A (supplementary material, IJSEM Online). Each of the five strains showed a distinct polar lipid profile, including the known polar lipids diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine as well as several unknown aminophospholipids, aminolipids, phospholipids and further polar lipids. The most pronounced characteristics of strain BN9^T were the presence of almost equal amounts of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and rather high amounts of the two characteristic aminolipids AL1 and AL2. In addition to the presence and absence of several minor lipids these characteristics clearly distinguished strain BN9^T from the other four strains. In contrast to a previous report of Yabuuchi *et al.* (1995), who described for *Alcaligenes faecalis* EY 1056^T only the presence of phosphatidylethanolamine (54%), phosphatidylglycerol (26%) and ornithine lipid OL-1 (20%), in the present study diphosphatidylglycerol and several unknown aminophospholipids, phospholipids, aminolipids and polar lipids were also detected [see

Fig. 1. Phylogenetic tree showing the relationship between strain BN9^T and selected strains from other bacterial taxa. The tree was constructed by using the maximum-parsimony method and the program TreeView. Bootstrap values based on 100 replications are given as percentages at the branching points. Bar, a difference of approximately 10 nt.

Table 1. Relative fatty acid compositions of strain BN9^T in comparison to species of the genera *Alcaligenes*, *Achromobacter*, *Pigmentiphaga* and *Kerstersia*

1, BN9^T; 2, *Alcaligenes faecalis* LMG 1229^T (data from Coenye *et al.*, 2003a); 3, *Alcaligenes defragrans* DSM 12141^T (this study); 4, *Achromobacter* (Coenye *et al.*, 2003a, b); 5, *Pigmentiphaga kullae* DSM 13608^T (Blümel *et al.*, 2001); 6, *Kerstersia gyiorum* LMG 5906^T (Coenye *et al.*, 2003a). Data given for the genus *Achromobacter* indicate the range of values described for *Achromobacter xylosoxidans*, *Achromobacter denitrificans*, *Achromobacter piechaudii*, *Achromobacter insolitus* and *Achromobacter spanius* (Coenye *et al.*, 2003a, b). —, Not present; 3-OH C_{10:0}, 3-hydroxydecanoic acid; C_{12:0}, dodecanoic acid=lauric acid; 2-OH C_{12:0}, 2-hydroxydodecanoic acid; C_{14:0}, tetradecanoic acid=myristic acid; 2-OH C_{14:0}, 2-hydroxytetradecanoic acid; 3-OH C_{14:0}, 3-hydroxytetradecanoic acid; C_{16:1}ω7c, *cis*-9-hexadecenoic acid=palmitoleic acid; C_{16:0}, hexadecanoic acid=palmitic acid; C_{17:0} cyclo, Δ-*cis*-9,10-methylene-hexadecanoic acid; 2-OH C_{16:0}, 2-hydroxyhexadecanoic acid; C_{18:1}ω7c, *cis*-11-octadecenoic acid=vaccenic acid; C_{18:0}, octadecanoic acid=stearic acid; C_{19:0} cyclo ω8c, Δ-*cis*-11,12-methylene-octadecanoic acid; Tr, trace amounts present. The fatty acid profiles of strains BN9^T and *Alcaligenes defragrans* DSM 12141^T were determined according to Kämpfer & Kroppenstedt (1996). Values characteristic for strain BN9^T are given in bold type.

Characteristic	1	2	3	4	5	6
C _{10:0}	Tr	—	Tr	—	—	—
Summed feature 1	1		3			
C _{12:0}	4	2	6	Tr	—	—
2-OH C _{12:0}	3	2	—	3–5	—	Tr
C _{13:1} AT 12–13	Tr	—	—	—	—	—
Summed feature 2	8*	10*	5	9–13*	5*	12*
Summed feature 3	Tr†	7†	28	5–18	—	4†
C _{16:0}	19	37	32	29–40	40	34
C _{17:0} cyclo	31	29	7	17–32	22	24
2-OH C _{16:0}	Tr	—	—	Tr–2·4	5	—
3-OH C _{16:0}	Tr	—	—	—	—	—
C _{18:1} ω7c	Tr	4	18‡	2–5	10§	11
C _{18:1} ω7c 11 methyl	Tr	—	—	—	—	—
C _{18:0}	3	Tr	—	2	—	3
C _{19:0} iso	Tr	—	Tr	—	—	—
C _{19:0} cyclo ω8c	27	Tr	Tr	Tr	12	2
C _{20:2} ω6,9c	Tr	—	Tr	—	—	—

*C_{16:1} iso I and/or 3-OH C_{14:0}.

†C_{16:1}ω7c and/or 2-OH C_{15:0} iso.

‡Given as C_{18:1}ω7c or C_{18:1}ω9t.

§Given as summed feature 7 (C_{18:1}ω7c, C_{18:1}ω9t and/or C_{18:1}ω12t).

supplementary Fig. A(II) in IJSEM Online]. *Achromobacter denitrificans* DSM 30026^T and *Achromobacter xylosoxidans* KM543^T were more closely related in their polar lipid profile [see supplementary Fig. A(IV) and (V) in IJSEM Online] to each other than to any other strain studied here, although they differed with regard to the presence of phospholipid PL6 and aminophospholipid 3 and with regard to the

Table 2. Polar lipid profiles in different members of the *Alcaligenaceae*

1, *Alcaligenes faecalis* DSM 30030^T; 2, *Achromobacter xylosoxidans* KM543^T; 3, *Achromobacter denitrificans* DSM 30026^T; 4, *Alcaligenes defragrans* DSM 12141^T; 5, BN9^T. DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; AL1–7, unknown aminolipids; PL1–6, unknown phospholipids; APL1–5, unknown aminophospholipids; L1–8, unknown polar lipids. + + + +, Major amounts; + + +, moderate amounts; + +, minor amounts; +, trace amounts; —, not detected.

Polar lipid	1	2	3	4	5
PE	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
PG	+ + +	+ +	+ +	+ + +	+ + + +
DPG	+ + +	+ + +	+ + +	+ +	+ + + +
AL1	+ +	+ +	+ +	+ +	+ + +
AL2	—	+	+	—	+ + + +
AL3	—	—	—	+ +	—
AL4	+ +	—	—	+ + +	—
AL5	+	—	—	+	—
AL6	+	+	+	+ +	—
AL7	+	—	—	—	—
PL1	—	—	—	+	—
PL2	+	—	—	+	+
PL3	—	+ + +	+	—	—
PL4	—	+ +	+	—	—
PL5	+	+	+	—	—
PL6	—	+	—	—	—
APL1	+	+ +	+	+	—
APL2	—	—	—	+	—
APL3	—	—	+	—	—
APL4	+	—	—	—	+
APL5	+	—	—	—	—
L1	—	—	—	—	+
L2	—	—	—	—	+
L3	+	+	+	+	+ +
L4	—	—	—	—	+
L5	—	—	—	+	—
L6	—	—	—	+	—
L7	—	—	—	+	—
L8	—	—	—	—	—

relative proportion of phospholipid PL3. The differences observed between *Achromobacter xylosoxidans* KM543^T and *Achromobacter denitrificans* DSM 30026^T supported the view of Coenye *et al.* (2003a) that the two strains represent different species and not two subspecies of *Achromobacter xylosoxidans* as favoured by Yabuuchi *et al.* (1998). The polar lipid profile of *Alcaligenes defragrans* DSM 12141^T showed several unique characteristics [Table 2 and see Supplementary Fig. A(III), IJSEM Online] that might be useful for a future reclassification of this species within a novel genus of the family *Alcaligenaceae*.

Strain BN9^T did not produce acids aerobically from various

sugars and sugar alcohols tested. Of various *p*-nitrophenyl and *p*-nitroaniline derivatives only L-alanine-*p*-nitroanilide was hydrolysed (method according to Kämpfer *et al.*, 1991). Only simple aliphatic and aromatic organic acids were utilized as sources of carbon and energy (for details, see species description below).

The family *Alcaligenaceae* currently consists of the recognized affiliated genera *Alcaligenes*, *Achromobacter*, *Advenella*, *Bordetella* and *Pigmentiphaga*. However, according to 16S rRNA gene sequence comparisons the genera *Brackiella*, *Kerstersia*, *Oligella*, *Pelistega*, *Sutterella* and *Taylorella* should also be included in this family (Busse & Stolz, 2004; Willems *et al.*, 2002; Coenye *et al.*, 2005). Phylogenetically, these genera form together with strain BN9^T a homogeneous cluster separate from other taxa within the 'Betaproteobacteria'. The affiliation of strain BN9^T to this family was unambiguously demonstrated by 16S rRNA gene sequence comparisons and phylogenetic analyses. This affiliation was also confirmed based on the presence of ubiquinone Q-8, putrescine and 2-hydroxyputrescine in the polyamine pattern (Busse & Auling, 1988; Busse *et al.*, 1992), and large amounts of 16:0 and 17:0 cyclo fatty acids present in strain BN9^T.

Two subgroups can be defined within the *Alcaligenaceae* based on the G + C content of their genomic DNA. Members of the genera *Alcaligenes*, *Achromobacter* and *Pigmentiphaga* have G + C contents ranging from 54 to 69 mol%. In contrast, members of the genera *Oligella*, *Taylorella* and *Pelistega* have only relatively low G + C contents of 36.5–47.5 mol% (Busse & Stolz, 2004). The G + C content of strain BN9^T was previously determined as 61.8 ± 0.2 mol% (Busse *et al.*, 1992) and thus would clearly suggest a closer relationship of strain BN9^T with the free-living members of the genera *Alcaligenes*, *Achromobacter* and *Pigmentiphaga* than with the genera *Oligella*, *Taylorella* and *Pelistega*. This was in clear contrast to the results of the 16S rRNA gene sequence comparisons, which placed strain BN9^T close to the 'low-G + C members' of the *Alcaligenaceae*.

Thus, strain BN9^T could not be affiliated to any of the recognized genera within the family *Alcaligenaceae*. Furthermore, 16S rRNA gene sequence similarity values between strain BN9^T and all other described species within the family *Alcaligenaceae* were ≤ 96 %. It was previously stated that 16S rRNA gene sequence similarity values among *Achromobacter* and *Bordetella* species are 97–98 % and that it could be assumed from these values that the threshold for the differentiation of genera within the family *Alcaligenaceae* is at < 97 % similarity (Blümel *et al.*, 2001). Strain BN9^T was also clearly separated from all other members of the *Alcaligenaceae* based on the presence of trace amounts of 3-OH C_{16:0} and by the large amounts of the fatty acid C_{19:0} cyclo ω8c. On the basis of these results, we consider that strain BN9^T is representative of a new taxon, for which the name *Pusillimonas noertemannii* gen. nov., sp. nov. is proposed.

Description of *Pusillimonas* gen. nov.

Pusillimonas (Pu.sil.li.mo' nas. L. adj. *pusillus* very small/minute; Gr. fem. n. *monas* unit/monad; N.L. fem. n. *Pusillimonas* very small monad/unicell, referring to the small size of cells and colonies of the type species).

Cells are Gram-negative, oxidase-positive and have an oxidative metabolism. G + C content of the DNA is 61.8 mol%. *Pusillimonas* belongs to the family *Alcaligenaceae*. The major isoprenoid quinone is ubiquinone Q-8. Putrescine, spermidine and 2-hydroxyputrescine are the major polyamines. Major polar lipids are two unknown aminolipids, phosphatidylglycerol, diphosphatidylglycerol and phosphatidylethanolamine. Major non-polar fatty acids are C_{17:0} cyclo, C_{19:0} cyclo ω8c, C_{16:0} and summed feature 2 (C_{16:1} iso I/3-OH C_{14:0}).

The type species is *Pusillimonas noertemannii*.

Description of *Pusillimonas noertemannii* sp. nov.

Pusillimonas noertemannii (noer.te.mann'i.i. N.L. gen. n. *noertemannii* of Nörtemann, in honour of Bernd Nörtemann, who isolated this and various other bacterial strains that had extraordinary degradative abilities).

The description of the species is as given for the genus above. Cells are rod-shaped (1–1.5 µm in length and 0.5–0.8 µm in diameter). They are motile by means of polarly inserted flagella. On nutrient agar, colonies are brownish, circular and with an entire margin. Grows at 30, 37 and 42 °C, but not at 4 °C. L-Alanine-*p*-nitroanilide is hydrolysed on the basis of the method described by Kämpfer *et al.* (1991). The following compounds are not hydrolysed: *p*-nitrophenyl β-D-galactopyranoside, *p*-nitrophenyl β-D-glucuronide, *p*-nitrophenyl α-D-glucopyranoside, *p*-nitrophenyl β-D-glucopyranoside, *p*-nitrophenyl β-D-xylopyranoside, bis-*p*-nitrophenyl phosphate, bis-*p*-nitrophenyl-phenyl phosphonate, bis-*p*-nitrophenyl-phosphoryl choline, L-aniline-*p*-nitroanilide, γ-L-glutamate-*p*-nitroanilide and L-proline-*p*-nitroanilide. The following compounds are used as sole source of carbon: acetate, propionate, DL-3-hydroxybutyrate, DL-lactate, 2-oxoglutarate, pyruvate and 4-hydroxybenzoate, on the basis of the method described by Kämpfer *et al.* (1991). The following compounds are not assimilated: *N*-acetylgalactosamine, *N*-acetylglucosamine, L-arabinose, L-arbutin, D-cellobiose, D-fructose, D-galactose, gluconate, D-glucose, D-maltose, D-mannose, α-D-melibiose, L-rhamnose, D-ribose, D-sucrose, salicin, D-trehalose, D-xylose, adonitol, *i*-inositol, maltitol, D-mannitol, D-sorbitol, putrescine, *cis*-aconitate, *trans*-aconitate, adipate, 4-aminobutyrate, azelate, citrate, fumarate, glutarate, itaconate, L-malate, mesaconate, suberate, L-alanine, L-aspartate, L-histidine, L-leucine, L-ornithine, L-phenylalanine, L-serine, L-tryptophan, 3-hydroxybenzoate and phenylacetate. No acids are produced from glucose, lactose, sucrose, D-mannitol, dulcitol, salicin, adonitol, inositol, sorbitol, L-arabinose, raffinose, rhamnose, maltose,

D-xylose, trehalose, cellobiose, methyl D-glucoside, erythritol, melibiose, D-arabitol or D-mannose. Quinone system, polyamine pattern and polar lipid profile are identical to those listed under the genus description. The fatty acid profile consists of C_{10:0} (trace), C_{12:0} (4.4 %), 2-OH C_{12:0} (3.3 %), C_{13:1} AT 12–13 (trace), summed feature 1 (C_{12:0} alde?) (1.2 %), summed feature 4 (C_{16:1} iso I and/or 3-OH C_{14:0}) (8.0 %), C_{16:1} ω 7c (trace), C_{16:0} (18.5 %), C_{17:0} cyclo (30.9 %), 2-OH C_{16:0} (trace), 3-OH C_{16:0} (trace), C_{18:1} ω 7c (trace), C_{18:1} ω 7c 11 methyl (trace), C_{18:0} (2.5 %), C_{19:0} iso (0.9 %), C_{19:0} cyclo ω 8c (26.8 %) and C_{20:2} ω 6,9c (trace).

The type strain (BN9^T=DSM 10065^T=NCIMB 14020^T) was isolated from the River Elbe in Germany after enrichment of a mixed bacterial culture with 6-aminonaphthalene-2-sulphonate. The G+C content of the DNA of the type strain is 61.8 mol% (*T*_m).

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