

# *Hydrocarboniphaga effusa* gen. nov., sp. nov., a novel member of the $\gamma$ -*Proteobacteria* active in alkane and aromatic hydrocarbon degradation

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Novel alkane-degrading strains of bacteria were isolated from soil contaminated with fuel oil from a leaking underground tank in New Jersey, USA. Two phenotypically similar strains (designated AP102 and AP103<sup>T</sup>) possessed 16S rRNA sequences unique among the majority of known hydrocarbon-degrading bacteria. The 16S rRNA sequences showed a moderate but distant relationship to the genus *Nevskia* and a substantial similarity to strains that had previously been isolated for growth on phenol (in Japan) and on toluene (in Canada) by other researchers. The hydrocarbon-degrading strains from Japan, Canada and New Jersey showed no resemblance to the typical morphology of *Nevskia* but did share a striking similarity among themselves in cell morphology, in the unusual appearance of colonies on various solid media and in various physiological properties. A full taxonomic analysis was performed, including DNA–DNA hybridization and nutritional screening with 117 organic compounds as sole sources of carbon and energy. The strains are active in the degradation of important environmental pollutants, and their phenotypic, physiological, metabolic and genomic properties suggest that they are members of a novel taxon in the  $\gamma$ -*Proteobacteria*, for which the name *Hydrocarboniphaga* gen. nov. is proposed, with the single species *Hydrocarboniphaga effusa* sp. nov. The type strain is AP103<sup>T</sup> (= ATCC BAA-332<sup>T</sup> = DSM 16095<sup>T</sup>).

## INTRODUCTION

Aliphatic and aromatic hydrocarbons are widely prevalent in the environment, the products of natural chemical and physical processes as well as a wide variety of human activities. In addition to natural oil seeps, petroleum and its derivatives are spread throughout the environment via the retrieval, transport, refining and use of fossil fuels, resulting in environmental pollution. It is not surprising therefore that some micro-organisms have adapted to utilize hydrocarbons as their sole sources of carbon and energy for growth. For instance, bacteria of many different genera are known to have the ability for aerobic and anaerobic microbial processes of alkane metabolism.

The four strains of bacteria described here were present in natural materials contaminated with hydrocarbons, and were isolated in three laboratories located distantly from one another. The strains represent a species belonging to

the  $\gamma$ -*Proteobacteria*, with a moderate 16S rRNA sequence similarity to the genus *Nevskia*. This paper gives a description of their properties, from which it is concluded that they are representative of a new genus that we propose to name *Hydrocarboniphaga* gen. nov. The genomic similarities among the strains suggest that they constitute a single species, which we name *Hydrocarboniphaga effusa* sp. nov.

## METHODS

**Bacterial strains and culture media.** Strains AP102 and AP103<sup>T</sup> were isolated from a soil sample from New Jersey contaminated with heavy fuel oil through the classic enrichment culture technique (Rosenberg, 1992), with dodecane provided in the gas phase as the sole carbon source. For the work described below, only strain AP103<sup>T</sup> was examined, because the two strains had the same general phenotypic properties and their 16S rRNA genes have nearly identical sequences. Strain LX1 was isolated from a compost biofilter in Canada from a bacterial population active in toluene degradation (Juteau *et al.*, 1999). Strains rJ4 and rJ5 were obtained from a Japanese activated sludge, in an enrichment using phenol as the sole carbon source (Watanabe *et al.*, 1999).

The complex media used in our work were trypticase soy agar (Difco), nutrient agar (Difco) and brain heart infusion agar (Difco). The chemically defined medium used in the nutritional screening was described by Palleroni & Doudoroff (1972) and Palleroni (1984).

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The GenBank accession numbers for the 16S rRNA gene sequences of strains AP102 and AP103<sup>T</sup> are AY363244 and AY363245, respectively.

## Characterization of the strains

**Morphological observations.** Microscopic observations were made and photomicrographs taken with a Leitz Ortholux II microscope with phase-contrast optics. Staining of flagella followed the method of Leifson (1954).

**Physiological characterization.** Denitrification was determined as described by Stanier *et al.* (1966). The method of Sierra (1957) was followed for the determination of lipase (Tween 80 hydrolysis) and gelatinase production was tested according to the method of Skerman (1967). Nutritional screening was performed according to Stanier *et al.* (1966), using the chemically defined medium of Palleroni & Doudoroff (1972). Carbon substrates were added from filter-sterilized stock solutions to a final concentration of 0.1%. Some substrates (alkanes, phenol) were supplied in the gas phase using small tubes with a cotton wick and sealing the plate with Parafilm.

**DNA extraction and purification.** The procedure of Marmur (1961) was followed for the extraction and purification of DNA used in the determination of DNA base composition and in DNA–DNA hybridization experiments.

**Determination of DNA base composition and of DNA–DNA relatedness.** Base composition of the purified DNA was determined by the method of Mesbah *et al.* (1989). For DNA–DNA hybridization experiments, the S1 nuclease procedure described by Johnson (1994a) was followed. DNA samples of the reference strains were labelled using [<sup>3</sup>H]thymidine and the nick translation procedure, following the instructions given by the kit manufacturer (Invitrogen).

**Amplification, cloning and sequencing of 16S rRNA genes.** Genomic DNA was isolated using a bacterial total genomic extraction kit (Clontech). The 16S rRNA genes were amplified using the 27f and 1522r primers by PCR under standard conditions as recommended by the supplier (Perkin Elmer). The PCR product was purified (Qiagen) and used directly in DNA sequencing reactions (Applied Biosystems) with the primers 27f, 357f, 704f, 926f, 1242f, 342r, 685r, 907r, 1392r and 1522r. Primer sequences are as described

by Johnson (1994b). The nucleotide sequences were aligned using CLUSTAL V in the Lasergene software package (DNASTar) and visually inspected. Phylogenetic analysis was performed with the MEGA version 2.1 software package (Kumar *et al.*, 2001). Distances (according to the Kimura two-parameter model) and clustering with the neighbour-joining and maximum-parsimony methods were determined using bootstrap values based on 1000 replications.

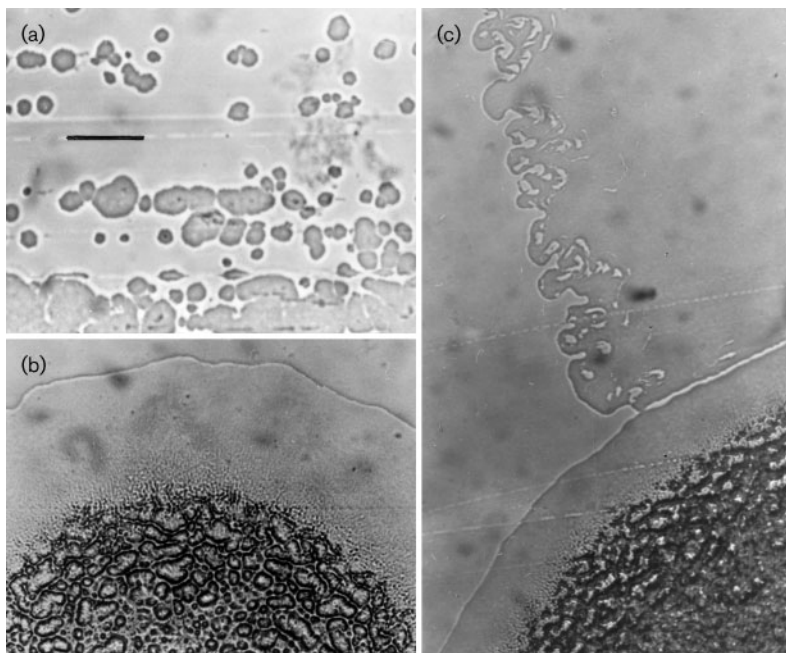
## RESULTS AND DISCUSSION

### Morphology and colony characteristics

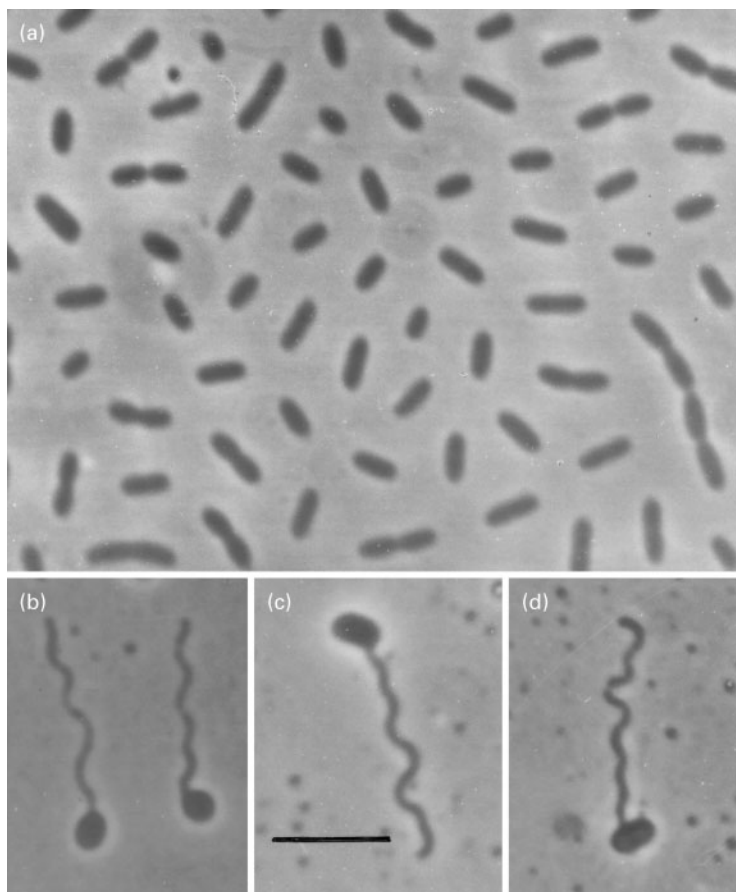
The four strains (AP103<sup>T</sup>, LX1, rJ4 and rJ5) were very similar in their basic morphological characteristics. The cells were small, Gram-negative rods, 0.75–0.85 × 1.5–2 µm (mean 0.8 × 1.2 µm) in size, occurring singly or rarely in short chains. They were motile by means of a single flagellum of polar, subpolar or, more rarely, lateral insertion (Fig. 1). Motility was moderate, usually limited to a few cells in the suspension.

Colonies on different agar media were of irregular shape and smooth when small, but later they became circular, with a wrinkled and folded dome at the centre and a thin margin of somewhat irregular border. The wrinkled appearance appears to be due to the presence of an extracellular product, possibly identical to a capsular material that prevented cell-to-cell contact in dense areas of wet mounts (Fig. 2). The chemical nature of this material has not been investigated.

The colonies were convex with flat edges, where the cells tended to swarm on the agar surface – hence the specific name *effusa*. Flat colonies composed of cells that swarmed more extensively were produced spontaneously. The extent



**Fig. 1.** Colonies of strain AP103<sup>T</sup>. (a) Young colonies. (b) Appearance of most colonies near the edge, after 4–5 days of incubation at 28 °C while growing on minimal medium at the expense of *n*-dodecane. (c) Flat variant colony growing at the edge of a normal colony, showing details of the edge. Bar, 5 mm.



**Fig. 2.** Cells of strain AP103<sup>T</sup>. (a) Phase-contrast. (b–d) Details of flagellar insertion: polar (predominating type) (b), subpolar (c), lateral (d). Bar, 5  $\mu$ m.

of swarming varied with the carbon source when the cells were growing on the chemically defined medium. Under these conditions, the production of the flat colony type was relatively common, and a reversion to the normal form was not observed. Twitching motility as the cause of swarming was not investigated.

### Physiological properties

The four strains were absolutely aerobic. The optimal temperature of growth was about 28 °C, with no significant growth seen above about 35 °C. All strains grew on complex media, such as trypticase soy agar, LB medium, nutrient agar and brain heart infusion agar, although growth on the last three media was much slower than on the first. However, the viability of the cells declined rapidly on trypticase soy agar. Viability was considerably longer on a minimal medium recommended for *Pseudomonas* (Palleroni & Doudoroff, 1972; Palleroni, 1984) when supplemented with each of a number of carbon sources (see below). Growth on this medium also indicates that organic growth factors are not required.

Ammonium salts or nitrate could be used as nitrogen sources. Denitrification was negative, lipase (Tween 80 hydrolysis) was positive (strains AP103<sup>T</sup> and LX1 gave a stronger reaction) and gelatinase was positive for AP103<sup>T</sup> and LX1 and very weak for rJ4 and rJ5. The four strains

were sensitive to ampicillin, tetracycline and kanamycin and moderately sensitive to chloramphenicol.

Nutritional spectra are given below under the species description. Table 1 summarizes the results obtained with additional substrates that are not utilized by all the strains and are therefore of interest for typing purposes. It is of note that the strains from Japan (rJ4 and rJ5) were nutritionally more versatile, because they grew at the expense of phenol and a number of carbohydrates, among them glucose and saccharides of glucose. Strains AP103<sup>T</sup> and LX1 were negative for these properties. Growth on sucrose by the latter was weak, presumably due to the utilization of the fructose moiety only. An additional difference was seen in the intensities of the gelatinase and lipase (Tween 80 hydrolysis) reactions, which were stronger for AP103<sup>T</sup> and LX1 than for rJ4 and rJ5.

### DNA base composition and DNA–DNA hybridization experiments

The DNA base composition of strain AP103<sup>T</sup> was 60 mol% and that of strain rJ5 was 61 mol%. On the basis of these values, and including formamide in the reaction mixture (25  $\mu$ l in a total volume of 110  $\mu$ l), the reannealing temperature in the DNA–DNA hybridization experiments was set as 66.3 °C. In these experiments, the DNA–DNA relatedness of AP103<sup>T</sup>, LX1, rJ4 and rJ5 to AP103<sup>T</sup> was 100,

**Table 1.** Differences in nutritional properties among the strains

+, Growth; -, no growth; ±, faint growth after a longer incubation time.

Substrate	AP103 <sup>T</sup>	LX1	rJ4	rJ5
Adonitol	±	±	+	+
D-Arabinose	-	-	±	±
L-Asparagine	±	±	+	+
L-Aspartate	±	±	+	+
Cellobiose	-	-	+	+
Glucose	-	-	+	+
Glycerol	±	±	+	+
L-Histidine	±	±	+	+
Mannose	±	±	+	+
Phenol	-	-	+	+
Propionate	+	+	-	-
Raffinose	-	-	+	+
Sucrose	±	±	+	+
Trehalose	-	-	+	+
Xylose	-	-	±	±

85, 84 and 78 %, respectively, and to rJ5 was 80, 90, 92 and 100 %, respectively.

**Phylogenetic analysis**

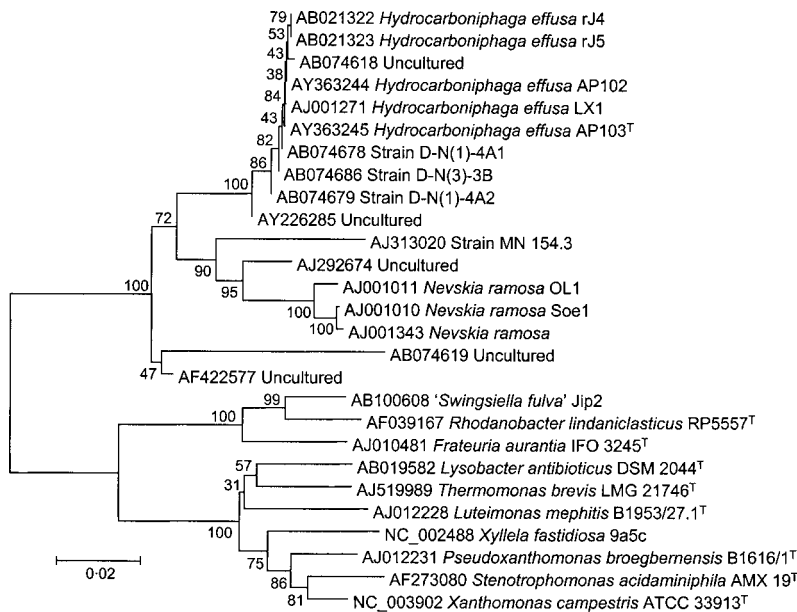
Near-complete (> 1450 nt) 16S rRNA gene sequences were determined for AP103<sup>T</sup> and AP102. A comparison of the 16S rRNA gene sequences for AP103<sup>T</sup>, AP102, rJ4, rJ5 and LX1 revealed that they differed in only six locations out of the 1403 positions that were shared among all five sequences. The closest characterized 16S rRNA gene sequence relatives in the GenBank database are those for *Nevskia ramosa* (Sturmeyer *et al.*, 1998). The three known *N. ramosa*

16S rRNA gene sequences share approximately 90 % similarity to the five *H. effusa* sequences. Interestingly, the *H. effusa* and *N. ramosa* 16S rRNA sequences clustered together and represent a deeply branching lineage in the *Xanthomonadaceae* (Fig. 3).

**Summary**

The strains described in this communication are representative of a new genus, for which we propose the name *Hydrocarboniphaga*, with the single species *Hydrocarboniphaga effusa*. The closest known relative based on 16S rRNA sequence similarity (about 90 %) is *Nevskia* in the  $\gamma$ -*Proteobacteria*. However, *Hydrocarboniphaga* strains can easily be distinguished from *Nevskia* strains due to the latter's propensity to form rosette-like colonies at air-water interfaces (Sturmeyer *et al.*, 1998). There is also a significant difference in the G + C content of strains of *Nevskia* (about 68 mol%) and *Hydrocarboniphaga* (60 mol%) (Sturmeyer *et al.*, 1998). Nutritionally, *Hydrocarboniphaga* strains grow on malate and succinate but not acetate, whereas *Nevskia* strains do not grow on malate or succinate and do grow on acetate (Sturmeyer *et al.*, 1998).

Although the four strains characterized here were all isolated for their ability to grow on hydrocarbons, 16S rRNA gene sequence comparisons indicate that *H. effusa* has a wide habitat. Three *H. effusa*-like strains were isolated from sediment from Lake Biwa (Japan) with selection for antibiotic resistance (Miyake *et al.*, 2003; AB074678, AB074679 and AB074686 in Fig. 3). Two 16S rRNA gene sequences from uncultured micro-organisms are included in the dendrogram in Fig. 3. One of these, AY226285, is from a deep-sea hypersaline anoxic basin in the Mediterranean Sea and the other, AB074618, is from a microbial community in the aposymbiotic pea aphid *Acyrtosiphon pisum* (Nakabachi *et al.*, 2003).



**Fig. 3.** Unrooted neighbour-joining dendrogram derived from 16S rRNA gene sequences showing phylogenetic relationships of AP103<sup>T</sup> and other *Hydrocarboniphaga effusa* strains, *Nevskia ramosa* strains and other representative strains in the *Xanthomonadaceae*. Bootstrap percentages at each node indicate the occurrence in 1000 bootstrapped trees. Bar, genetic distance of 0.02.

## Description of *Hydrocarboniphaga* gen. nov.

*Hydrocarboniphaga* (Hy.dro.car'bon.i.pha.ga. N.L. n. *hydrocarbonum* hydrocarbon; Gr. n. *phagos* eater, devourer; N.L. fem. n. *Hydrocarboniphaga* eater of hydrocarbons).

Gram-negative rods, 0.75–0.85 × 1.5–2 µm (mean 0.8 × 1.2 µm), occurring singly or rarely in short chains, motile by means of a single flagellum of polar or subpolar insertion (Fig. 1). On solid culture media, particularly on those chemically defined, swarming cells at the border cover a large area. The cells are absolutely aerobic and grow well on complex media and on chemically defined media, indicating that they do not require organic growth factors. Ammonium salts or nitrate can be used as nitrogen sources. Denitrification is negative. Gelatinase and lipase (Tween 80 hydrolysis) are positive. The G+C content of the DNA is 60–61 mol% (determined by HPLC). The type and only species is *Hydrocarboniphaga effusa*.

## Description of *Hydrocarboniphaga effusa* sp. nov.

*Hydrocarboniphaga effusa* (ef.fu'sa. L. adj. *effusa* spreading, diffuse, making reference to the spreading tendency of colonies growing on minimal agar medium).

General morphology and physiological characteristics are given above under the genus description. All strains grow on aliphatic hydrocarbons from C6 to C19, amyl alcohol, L-arginine, 2,3-butylene glycol, erythritol, ethanol, fumarate, L-glutamate, L-isoleucine, DL-lactate, L-leucine, L-malate, mannitol, L-phenylalanine, propylene glycol, pyruvate, L-rhamnose, succinate and L-valine. Growth on butyrate, caprate, caproate and caprylate is slow and very poor. None of the strains uses the following for growth: acetate, aconitate, adipate, L-alanine,  $\alpha$ -aminobutyrate,  $\delta$ -aminolaevulinate, amygdalin,  $\alpha$ -amylamine, anthranilate, arbutin, benzoate, betaine, *n*-butanol, L-citrulline, creatine, creatinine, dipicolinate, dulcitol, ethylene glycol, D-fucose, galactose, gluconate, glucosamine, glucuronate, histamine, *m*-, *o* or *p*-hydroxybenzoate, 2-ketogluconate, 2-ketogulonate, 2-propanol, L-kynurenine, L-lysine, L-lyxose, malonate, maltose, D- or L-mandelate, melibiose, methyl  $\alpha$ -glucoside, methyl  $\alpha$ -mannoside, mucate, naphthalene, nicotinate, norleucine, norvaline, pantothenate, L-proline, quinate, saccharate, sarcosine, sebacate, L-serine, sorbose, spermine, D-, *meso*- or L-tartrate, terephthalate, L-threonine, *o*-, *m*- or *p*-toluate, turanose or xylitol.

The type strain is AP103<sup>T</sup> (=ATCC BAA-332<sup>T</sup>=DSM 16095<sup>T</sup>).

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