

## Reclassification of *Aquaspirillum itersonii* and *Aquaspirillum peregrinum* as *Novispirillum itersonii* gen. nov., comb. nov. and *Insolitospirillum peregrinum* gen. nov., comb. nov.

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Phylogenetic analysis based on 16S rRNA gene sequences showed that *Aquaspirillum itersonii* and *Aquaspirillum peregrinum* form distinct phylogenetic lineages within the *Alphaproteobacteria*, whereas *Aquaspirillum serpens*, the type species of the genus *Aquaspirillum*, belongs to the *Betaproteobacteria*. *A. itersonii* and *A. peregrinum* exhibited 16S rRNA gene sequence similarity values of 82.0–82.4% to the type strain of *A. serpens* and of 91.8–92.0% to each other. *A. itersonii* and *A. peregrinum* were clearly distinguishable from *A. serpens* by differences in ubiquinone types and fatty acid profiles. *A. itersonii* subsp. *itersonii* LMG 4337<sup>T</sup> and *A. itersonii* subsp. *nipponicum* LMG 7370<sup>T</sup> contained Q-10 as the predominant ubiquinone, and *A. peregrinum* subsp. *peregrinum* LMG 4340<sup>T</sup> and *A. peregrinum* subsp. *integrum* LMG 5407<sup>T</sup> contained Q-9 as the predominant ubiquinone, whereas *A. serpens* LMG 3734<sup>T</sup> had Q-8 as the predominant ubiquinone. *A. itersonii* and *A. peregrinum* were also distinguishable from *A. serpens* by some differences in the fatty acid composition, including major fatty acids and hydroxy fatty acids. On the basis of these data, *A. itersonii* and *A. peregrinum* should be reclassified into two novel genera and species, for which the names *Novispirillum itersonii* gen. nov., comb. nov. and *Insolitospirillum peregrinum* gen. nov., comb. nov., respectively, are proposed.

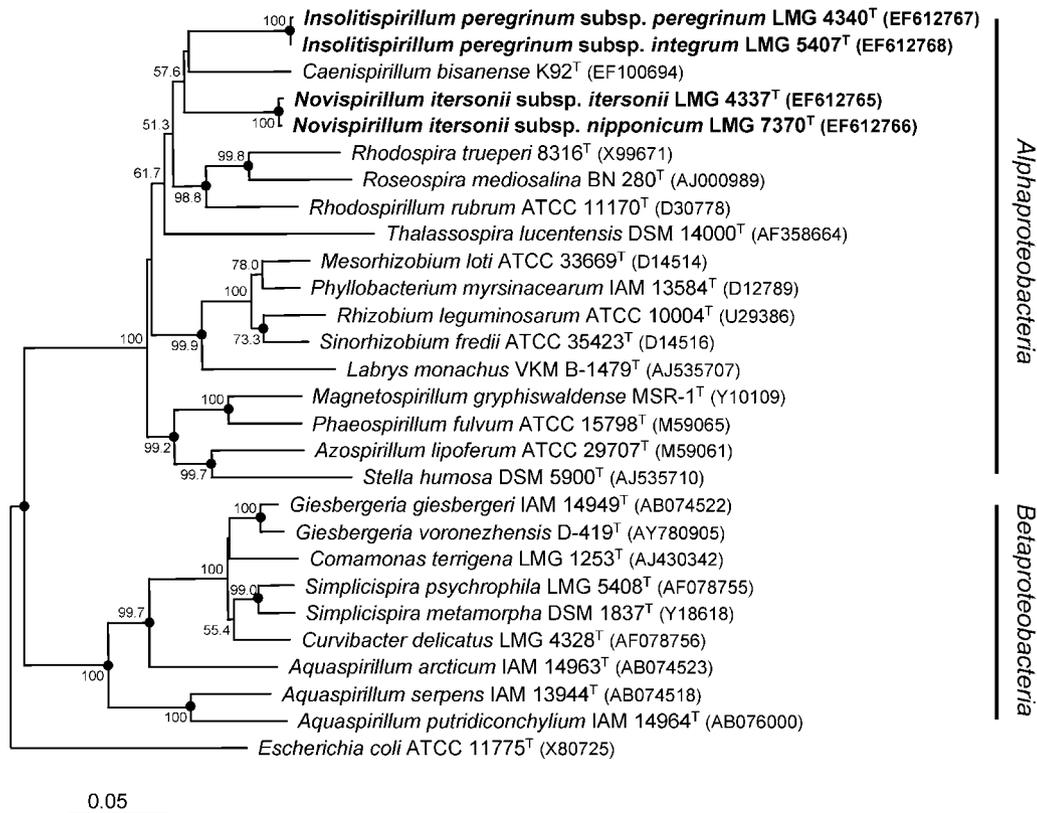
The genus *Aquaspirillum* was created by Hylemon *et al.* (1973) with the descriptions of 13 species and, subsequently, further *Aquaspirillum* species have been described (Kumar *et al.*, 1974; Strength *et al.*, 1976; Aragno & Schlegel, 1978; Maratea & Blakemore, 1981; Butler *et al.*, 1989). However, many *Aquaspirillum* species have been transferred to other genera or reclassified as members of novel genera (Schleifer *et al.*, 1991; Pot *et al.*, 1992; Cleenwerck *et al.*, 2003; Wauters *et al.*, 2003; Ding & Yokota, 2004; Spring *et al.*, 2004; Grabovich *et al.*, 2006). *Aquaspirillum itersonii* and *Aquaspirillum peregrinum* were also found to be phylogenetically related more closely to the *Alphaproteobacteria* than to the *Betaproteobacteria*, to which the type species of the genus *Aquaspirillum*, *Aquaspirillum serpens*, belongs. *A. itersonii* and *A. peregrinum* were described by Hylemon *et al.* (1973) as a result of the reclassification of ‘*Spirillum itersonii*’ (Giesberger,

1936) and ‘*Spirillum peregrinum*’ (Pretorius, 1963), respectively. ‘*S. itersonii* subsp. *nipponicum*’ and ‘*S. peregrinum* subsp. *integrum*’, described by Terasaki (1973), were reclassified as *A. itersonii* subsp. *nipponicum* and *A. peregrinum* subsp. *integrum* by Terasaki (1979). *A. itersonii* and *A. peregrinum* were placed into the genus *Aquaspirillum* on the basis of morphological, physiological and nutritional characteristics and DNA base compositions (Hylemon *et al.*, 1973; Terasaki, 1979). Accordingly, the aim of the present work was to determine the exact taxonomic positions of *A. itersonii* and *A. peregrinum* by a polyphasic characterization that included the determination of phenotypic and chemotaxonomic properties and a detailed phylogenetic analysis based on newly determined 16S rRNA gene sequences.

*A. itersonii* subsp. *itersonii* LMG 4337<sup>T</sup>, *A. itersonii* subsp. *nipponicum* LMG 7370<sup>T</sup>, *A. peregrinum* subsp. *peregrinum* LMG 4340<sup>T</sup>, *A. peregrinum* subsp. *integrum* LMG 5407<sup>T</sup> and *A. serpens* LMG 3734<sup>T</sup> were obtained from the Laboratorium voor Microbiologie Universiteit Gent (LMG), Gent, Belgium. To investigate their physiological and biochemical characteristics, *A. itersonii* and *A. peregrinum* strains were cultivated routinely at 28 °C in

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains LMG 4337<sup>T</sup>, LMG 7370<sup>T</sup>, LMG 4340<sup>T</sup> and LMG 5407<sup>T</sup> determined in this study are EF612765–EF612768, respectively.

Two-dimensional thin-layer chromatograms of polar lipids are available with the online version of this paper.



**Fig. 1.** Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of *Novispirillum* (= *Aquaspirillum*) *itersonii*, *Insolitispirillum* (= *Aquaspirillum*) *peregrinum* and related taxa. Bootstrap values (expressed as percentages of 1000 replications) >50% are shown at branch points. Dots indicate that the corresponding nodes were also recovered in trees generated with the maximum-likelihood and maximum-parsimony algorithms. *Escherichia coli* ATCC 11775<sup>T</sup> was used as an outgroup. Bar, 0.05 substitutions per nucleotide position.

LMG medium no. 8, which contained (l distilled water)<sup>-1</sup>: 1 g succinic acid, 10 g peptone, 1 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 2 mg FeCl<sub>3</sub>·6H<sub>2</sub>O, 2 mg MnSO<sub>4</sub>·H<sub>2</sub>O and 15 g agar, pH 7.0. Growth under anaerobic conditions was determined after incubation in a Forma anaerobic chamber on solid LMG medium no. 8 and on solid LMG medium no. 8 supplemented with potassium nitrate (0.1%, w/v), both of which had been prepared under a nitrogen atmosphere. Growth at various temperatures (4–45 °C) was measured on solid LMG medium no. 8. Catalase and oxidase activities and hydrolysis of casein, gelatin, hypoxanthine, starch, Tweens 20, 40, 60 and 80, tyrosine, urea and xanthine were determined as described by Cowan & Steel (1965). Aesculin hydrolysis and nitrate reduction were studied as described by Lanyi (1987). Susceptibility to antibiotics was tested on solid LMG medium no. 8 plates, using antibiotic discs containing the following antibiotics: polymyxin B, 100 U; streptomycin, 50 µg; penicillin G, 20 U; chloramphenicol, 100 µg; ampicillin, 10 µg; cephalothin, 30 µg; gentamicin, 30 µg; novobiocin, 5 µg; tetracycline, 30 µg; kanamycin, 30 µg; lincomycin, 15 µg; oleandomycin, 15 µg; neomycin, 30 µg;

carbenicillin, 100 µg. Assimilation of various substrates, enzyme activities and other physiological and biochemical properties were tested by using the API 20E, API 20NE, API 50CH and API ZYM systems (bioMérieux); assimilation of various substrates was determined by inoculating the API 50CH strips with cells suspended in AUX medium (bioMérieux).

Cell biomass for DNA extraction and for analyses of isoprenoid quinones and polar lipids was obtained from cultivation in liquid LMG medium no. 8 at 28 °C. Chromosomal DNA was isolated and purified according to the method described by Yoon *et al.* (1996), with the exception that RNase T1 was used in combination with RNase A to minimize RNA contamination. The 16S rRNA gene was amplified by PCR using two universal primers, 5'-GAGTTTGATCCTGGCTCAG-3' and 5'-AGAAAGG-AGGTGATCCAGCC-3', as described previously (Yoon *et al.*, 1998). Sequencing of the amplified 16S rRNA gene and phylogenetic analysis were performed as described previously (Yoon *et al.*, 2003). Isoprenoid quinones were analysed by the method of Komagata & Suzuki (1987), using reversed-phase HPLC. For cellular fatty acid analysis,

**Table 1.** Percentage cellular fatty acid compositions of *Aquaspirillum itersonii*, *Aquaspirillum peregrinum* and *Aquaspirillum serpens* from this study

Strains: 1, *A. itersonii* subsp. *itersonii* LMG 4337<sup>T</sup>; 2, *A. itersonii* subsp. *nipponicum* LMG 7370<sup>T</sup>; 3, *A. peregrinum* subsp. *peregrinum* LMG 4340<sup>T</sup>; 4, *A. peregrinum* subsp. *integrum* LMG 5407<sup>T</sup>; 5, *A. serpens* LMG 3734<sup>T</sup>. Fatty acids that represented <0.5% in all strains were omitted.

Fatty acid	1	2	3	4	5
<b>Straight chain</b>					
C <sub>12:0</sub>	3.6	3.7	2.9	3.0	4.4
C <sub>14:0</sub>	0.3	—	—	0.8	0.9
C <sub>15:0</sub>	—	—	—	—	0.8
C <sub>16:0</sub>	13.9	5.2	9.0	15.6	18.8
C <sub>17:0</sub>	—	—	—	—	0.6
C <sub>18:0</sub>	0.6	1.1	0.4	2.0	0.4
<b>Unsaturated</b>					
C <sub>12:1</sub>	—	—	8.0	—	—
C <sub>15:1<math>\omega</math>6c</sub>	—	—	—	—	0.9
C <sub>16:1<math>\omega</math>5c</sub>	1.7	0.8	1.8	1.3	0.2
C <sub>17:1<math>\omega</math>6c</sub>	0.2	0.2	—	—	0.9
C <sub>18:1<math>\omega</math>5c</sub>	0.7	1.9	1.0	1.5	—
C <sub>18:1<math>\omega</math>7c</sub>	54.2	67.2	49.2	53.9	14.5
<b>Hydroxy</b>					
C <sub>12:0</sub> 3-OH	—	—	—	—	5.5
C <sub>16:0</sub> 3-OH	2.8	2.6	2.5	2.3	—
C <sub>18:1</sub> 2-OH	0.5	4.1	6.7	0.4	—
C <sub>18:0</sub> 3-OH	1.1	1.6	0.4	0.8	—
<b>Unknown</b>					
ECL* 14.502	0.5	0.4	0.4	0.5	—
<b>Summed features†</b>					
2	2.8	3.0	3.0	2.8	—
3	16.7	7.9	14.5	15.2	51.4

\*ECL, Equivalent chain length.

†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 iso-C<sub>16:1</sub> and/or C<sub>14:0</sub> 3-OH. Summed feature 3 contained C<sub>16:1 $\omega$ 7c</sub> and/or iso-C<sub>15:0</sub> 2-OH.

cell biomass of the five strains was harvested from solid LMG medium no. 8 plates after incubation for 3 days at 28 °C. Fatty acids were extracted and fatty acid methyl esters were prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification system (Sasser, 1990). Polar lipids were extracted according to Minnikin *et al.* (1984) and identified by two-dimensional TLC after spraying with appropriate detection reagents (Minnikin *et al.*, 1984; Komagata & Suzuki, 1987). The DNA G+C content was determined by the method of Tamaoka & Komagata (1984), with a modification that DNA was hydrolysed by using nuclease P1 (Sigma) and the resultant nucleotides were analysed by reversed-phase HPLC.

Almost-complete 16S rRNA gene sequences of *A. itersonii* subsp. *itersonii* LMG 4337<sup>T</sup>, *A. itersonii* subsp. *nipponicum* LMG 7370<sup>T</sup>, *A. peregrinum* subsp. *peregrinum* LMG 4340<sup>T</sup>

and *A. peregrinum* subsp. *integrum* LMG 5407<sup>T</sup> determined in this study each comprised 1442 nt. There were five nucleotide differences in the sequences between *A. itersonii* subsp. *itersonii* LMG 4337<sup>T</sup> and *A. itersonii* subsp. *nipponicum* LMG 7370<sup>T</sup>. There were two nucleotide differences between *A. peregrinum* subsp. *peregrinum* LMG 4340<sup>T</sup> and *A. peregrinum* subsp. *integrum* LMG 5407<sup>T</sup>. In the phylogenetic tree, *A. itersonii* subsp. *itersonii* LMG 4337<sup>T</sup>, *A. itersonii* subsp. *nipponicum* LMG 7370<sup>T</sup>, *A. peregrinum* subsp. *peregrinum* LMG 4340<sup>T</sup> and *A. peregrinum* subsp. *integrum* LMG 5407<sup>T</sup> formed distinct phylogenetic lineages within the *Alphaproteobacteria* and were related distantly to the clade comprising *A. serpens*, the type species of the genus *Aquaspirillum*, which belongs to the *Betaproteobacteria* (Fig. 1). *A. itersonii* and *A. peregrinum* showed low 16S rRNA gene sequence similarity values of 91.8–92.0% to each other, and similarity values of 82.2–82.4 and 82.0% to the type strain of *A. serpens*, respectively.

The predominant isoprenoid quinone detected in *A. itersonii* subsp. *itersonii* LMG 4337<sup>T</sup> and *A. itersonii* subsp. *nipponicum* LMG 7370<sup>T</sup> was ubiquinone-10 (Q-10) at a peak area ratio of approximately 90–92%. The predominant isoprenoid quinone detected in *A. peregrinum* subsp. *peregrinum* LMG 4340<sup>T</sup> and *A. peregrinum* subsp. *integrum* LMG 5407<sup>T</sup> was ubiquinone-9 (Q-9) at a peak area ratio of approximately 89–90%. *A. serpens* LMG 3734<sup>T</sup> had Q-8 as the predominant ubiquinone, at a peak area ratio of approximately 95%. The same results were obtained by Sakane & Yokota (1994). Accordingly, *A. itersonii* and *A. peregrinum* could be distinguished clearly from *A. serpens* and from each other by differences in the predominant ubiquinone types. The cellular fatty acid profiles also distinguish *A. itersonii* and *A. peregrinum* from *A. serpens* (Table 1). *A. itersonii* and *A. peregrinum* contain C<sub>18:1 $\omega$ 7c</sub> as the major fatty acid, whereas *A. serpens* contains C<sub>16:1 $\omega$ 7c</sub> and/or iso-C<sub>15:0</sub> 2-OH (Table 1). *A. itersonii* and *A. peregrinum* were also distinguishable from *A. serpens* by some differences in fatty acid composition, including hydroxy fatty acids (Table 1), as also shown by the study of Sakane & Yokota (1994). The phylogenetic and chemotaxonomic data suggest that *A. itersonii* and *A. peregrinum* should be placed in two different genera that are distinct from the genus *Aquaspirillum*. Therefore, we propose to reclassify *A. itersonii* and *A. peregrinum* as two novel genera and species, *Novispirillum itersonii* gen. nov., comb. nov. and *Insolitispirillum peregrinum* gen. nov., comb. nov., respectively.

### Description of *Novispirillum* gen. nov.

*Novispirillum* (No'vi.spi.ril'lum. L. adj. *novus* new; N.L. dim. neut. n. *spirillum* a small spiral; N.L. neut. n. *Novispirillum* a new small spiral).

Cells are Gram-negative and helical. Catalase- and oxidase-positive. Nitrate is reduced to nitrogen gas. The predominant ubiquinone is Q-10. The major fatty acid is C<sub>18:1 $\omega$ 7c</sub>. The type species is *Novispirillum itersonii* (Giesberger 1936).

**Table 2.** Phenotypic characteristics of *Novispirillum* (= *Aquaspirillum*) *itersonii* and *Insolitisspirillum* (= *Aquaspirillum*) *peregrinum* determined in this study

Strains: 1, *N. itersonii* subsp. *itersonii* LMG 4337<sup>T</sup>; 2, *N. itersonii* subsp. *nipponicum* LMG 7370<sup>T</sup>; 3, *I. peregrinum* subsp. *peregrinum* LMG 4340<sup>T</sup>; 4, *I. peregrinum* subsp. *integrum* LMG 5407<sup>T</sup>. Data are from this study. +, Positive reaction; -, negative reaction; w, weakly positive reaction. All strains are positive for growth at 15 °C, catalase, oxidase, hydrolysis of aesculin, utilization of fructose, alkaline phosphatase, naphthol-AS-BI-phosphohydrolase and susceptibility to streptomycin, chloramphenicol, gentamicin, tetracycline, kanamycin and neomycin. All strains are negative for Gram staining, growth at 10 °C, hydrolysis of casein, gelatin, hypoxanthine, xanthine, starch and Tweens 20, 40, 60 and 80, utilization of erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, β-methyl-D-xyloside, galactose, glucose, mannose, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, α-methyl-D-mannoside, α-methyl-D-glucoside, N-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate, 5-ketogluconate, caprate, adipate, citrate, phenylacetate, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, N-acetylglucosaminidase, α-mannosidase, α-fucosidase and susceptibility to penicillin G, ampicillin, cephalothin and oleandomycin.

Characteristic	1	2	3	4
Maximum growth temperature (°C)	43	41	39	39
Nitrate reduction	+	+	-	-
Anaerobic growth with KNO <sub>3</sub>	+	+	-	-
Hydrolysis of:				
Tyrosine	+	+	-	-
Urea	-	-	+	+
Utilization of:				
Glycerol	-	+	-	-
Aesculin	-	-	+	+
Malate	+	+	-	-
Enzyme activity (by API ZYM):				
Esterase (C4)	+	+	-	-
Esterase lipase (C8)	+	+	-	-
Leucine arylamidase	w	-	-	-
Acid phosphatase	w	-	-	w
β-Glucosidase	-	-	+	+
Susceptibility to:				
Polymyxin B	w	-	+	+
Novobiocin	+	+	-	-
Carbenicillin	-	-	+	+
Lincomycin	-	+	-	-
Predominant ubiquinone	Q-10	Q-10	Q-9	Q-9
Major polar lipids*	PG, PE, AL	PG, PE, AL, L	PG, PE, AL, PL	PG, PE, AL, L
DNA G + C content (mol%)	63.2	64.7	62.4	62.3

\*PG, Phosphatidylglycerol; PE, phosphatidylethanolamine; AL, unidentified aminolipid; L, unidentified lipid; PL, unidentified phospholipid.

### Description of *Novispirillum itersonii* (Giesberger 1936) comb. nov.

*Novispirillum itersonii* (i.ter.so'ni.i. N.L. gen. n. *itersonii* named after G. Van Iterson, a Dutch bacteriologist).

Basonym: *Aquaspirillum itersonii* (Giesberger 1936).

The description is as that given by Hylemon *et al.* (1973) and Terasaki (1979). Characteristics of the type strain determined in this study are given in Table 2.

### Description of *Novispirillum itersonii* subsp. *itersonii* (Giesberger 1936) comb. nov.

*Novispirillum itersonii* subsp. *itersonii* (i.ter.so'ni.i. N.L. gen. n. *itersonii* named after G. Van Iterson, a Dutch bacteriologist).

Basonym: *Aquaspirillum itersonii* subsp. *itersonii* (Giesberger 1936) Hylemon *et al.* 1973.

The description is as that given by Hylemon *et al.* (1973). Characteristics of the type strain determined in this study

are given in Table 2. The type strain is ATCC 12639<sup>T</sup>=LMG 4337<sup>T</sup>=CCUG 49447<sup>T</sup>.

**Description of *Novispirillum itersonii* subsp. *nipponicum* (Terasaki 1973) comb. nov.**

*Novispirillum itersonii* subsp. *nipponicum* (nip.po'ni.cum. N.L. neut. adj. *nipponicum* pertaining to the country of Japan).

Basonym: *Aquaspirillum itersonii* subsp. *nipponicum* (Terasaki 1973) Terasaki 1979.

The description is as that given by Terasaki (1979). Characteristics of the type strain determined in this study are given in Table 2. The type strain is ATCC 33333<sup>T</sup>=LMG 7370<sup>T</sup>=CCUG 49448<sup>T</sup>.

**Description of *Insolitispirillum* gen. nov.**

*Insolitispirillum* (In.so.li'ti.spi.ril'lum. L. adj. *insolitus* unaccustomed; N.L. dim. neut. n. *spirillum* a small spiral; N.L. neut. n. *Insolitispirillum* an unaccustomed small spiral).

Cells are Gram-negative and helical. Catalase- and oxidase-positive. Nitrate is not reduced. The predominant ubiquinone is Q-9. The major fatty acid is C<sub>18:1ω7c</sub>. The type species is *Insolitispirillum peregrinum* (Pretorius 1963).

**Description of *Insolitispirillum peregrinum* (Pretorius 1963) comb. nov.**

*Insolitispirillum peregrinum* (pe.re.gri'num. L. neut. adj. *peregrinum* strange, foreign).

Basonym: *Aquaspirillum peregrinum* (Pretorius 1963) Hylemon *et al.* 1973.

The description is as that given by Hylemon *et al.* (1973) and Terasaki (1979). Characteristics of the type strain determined in this study are given in Table 2.

**Description of *Insolitispirillum peregrinum* subsp. *peregrinum* (Pretorius 1963) comb. nov.**

*Insolitispirillum peregrinum* subsp. *peregrinum* (pe.re.gri'num. L. neut. adj. *peregrinum* strange, foreign).

Basonym: *Aquaspirillum peregrinum* subsp. *peregrinum* (Pretorius 1963) Hylemon *et al.* 1973.

The description is as that given by Hylemon *et al.* (1973). Characteristics of the type strain determined in this study are given in Table 2. The type strain is ATCC 15387<sup>T</sup>=LMG 4340<sup>T</sup>=CCUG 13795<sup>T</sup>=DSM 1839<sup>T</sup>.

**Description of *Insolitispirillum peregrinum* subsp. *integrum* (Terasaki 1973) comb. nov.**

*Insolitispirillum peregrinum* subsp. *integrum* (in'te.grum. L. neut. adj. *integrum* unchanged, referring to failure to form coccoid bodies).

Basonym: *Aquaspirillum peregrinum* subsp. *integrum* (Terasaki 1973) Terasaki 1979.

The description is as that given by Terasaki (1979). Characteristics of the type strain determined in this study are given in Table 2. The type strain is ATCC 33334<sup>T</sup>=LMG 5407<sup>T</sup>=CCUG 49449<sup>T</sup>=DSM 11589<sup>T</sup>.

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