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Reclassification of *Serpens flexibilis* Hespell 1977 as *Pseudomonas flexibilis* comb. nov., with *Pseudomonas tuomuerensis* Xin *et al.* 2009 as a later heterotypic synonym

Su-Kyoung Shin [a], Chung Yeon Hwang [b], Yong-Joon Cho [c], Hana Yi [a,d,*]

[a] *BK21PLUS Program in Embodiment: Health-Society Interaction, Department of Public Health Sciences, Graduate School, Korea University, Seoul, Republic of Korea*
[b] *Division of Polar Life Sciences, Korea Polar Research Institute, Incheon, Republic of Korea*
[c] *ChunLab, Inc., Seoul, Republic of Korea*
[d] *School of Biosystem and Biomedical Science, Korea University, Seoul, Republic of Korea*

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ABSTRACT

Serpens flexibilis was proposed in 1977 and approved in 1980 without the 16S rRNA gene sequence information. The sequence of *S. flexibilis* became available in 2010, after the publication of *Pseudomonas tuomuerensis* in 2009. Our preliminary phylogenetic analyses indicated that these two strains share high sequence similarity and therefore showed strong potential to be united into a single species. To clarify the taxonomic status of the two species, a polyphasic taxonomy study was conducted including whole genome sequencing. The value of average nucleotide identity (ANI) and digital DNA-DNA hybridization (dDDH) between the genome sequences of *S. flexibilis* ATCC 29606^T and *P. tuomuerensis* JCM 14085^T were 98.1% and 89.0%, respectively. The phenotypic and chemotaxonomic properties including enzymatic activities, substrate utilization profiles, and fatty acids, supported that the two taxa have no pronounced difference and should thus constitute a single species. Therefore, we propose to transfer *Serpens flexibilis* Hespell 1977 to the genus *Pseudomonas* as *Pseudomonas flexibilis* comb. nov. (type strain = ATCC 29606^T), with *Pseudomonas tuomuerensis* Xin *et al.* 2009 as a later heterotypic synonym of *Pseudomonas flexibilis*.

The GenBank/EMBL/DDBJ accession number for the genome sequence of *Serpens flexibilis* ATCC 29606^T and *Pseudomonas tuomuerensis* JCM 14085^T are JRUD01000000 and JTAK00000000, respectively.

The type strain of *Serpens flexibilis* was initially isolated from pond mud and the species description was reported based on morphological and physiological characteristics [11]. The name was listed on the Approved Lists of Bacterial Names in 1980 [26]. Subsequently, according to the 16S rRNA oligonucleotide cataloging technique, *S. flexibilis* was shown to be most closely related to *Pseudomonas pseudoalcaligenes* among different genera of helical bacteria, and thus was presumed as a variant pseudomonad [32]. Further investigation using the same technique revealed that *S. flexibilis* was closely related to the subgroup Ia pseudomonads [31]. Analysis of aromatic amino acid biosynthesis enzymes suggested that *S. flexibilis* belonged to the cluster containing *P. stutzeri*, *P. mendocina*, *P. alcaligenes*, and *P. pseudoalcaligenes* within the subgroup Ia pseudomonads [1]. Nevertheless, until the 16S rRNA gene sequence of the type strain was released in 2010 (GenBank accession number, GU269546), the conflicting taxonomic status of the genera *Serpens* and *Pseudomonas* had not been clearly identified.

The genus *Pseudomonas*, proposed by Migula [19], comprises a group of Gram-negative bacteria that are rod-shaped, non-spore-forming, strictly aerobic, and motile by polar flagella [21]. This genus is one of the most diverse and ubiquitous bacterial genera whose species have been isolated from all kinds of environments such as soils, water, plants, clinical specimens, and marine habitats [22,25]. This genus is highly heterogeneous and has undergone several reclassifications on the basis of phenotypic, chemotaxonomic, and genetic studies [2,20,29].

P. tuomuerensis, with a type strain isolated from a bird's nest, was proposed as a novel species of the genus *Pseudomonas* based on phylogenetic trees, phenotypic characteristics, and DNA-DNA relatedness data [33]. The closest relatives were reported as *P. mendocina*, *P. pseudoalcaligenes*, and *P. alcaliphila* (16S rRNA gene

Abbreviations: ANI, average nucleotide identity; dDDH, digital DNA-DNA hybridization.

* Corresponding author at: School of Biosystem and Biomecial Science, Korea University, Seoul, Republic of Korea. Tel.: + 82 2 3290 5644.
E-mail address: hanayi@korea.ac.kr (H. Yi).

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Table 1
Genomic statistics of *Serpens flexibilis* ATCC 29606^T and *Pseudomonas tuomuerensis* JCM 14085^T.

	S. flexibilis		*P. tuomuerensis*	
Attribute	Value	% of total	Value	% of total
Genome size (bp)	3,762,694	100	3,748,402	100
DNA coding (bp)	3,363,168	89.4	3,363,168	89.7
DNA G + C (bp)	2,475,476	65.8	2,467,573	65.8
Total genes	3,534	100	3,489	100
Protein coding genes	3,454	97.7	3,417	97.9
RNA genes	66	1.9	65	1.9
Pseudo genes	14	0.4	7	0.2
Genes with function prediction	2,422	68.5	2,451	70.2
Genes assigned to COGs	2,923	82.7	2,929	83.9
Genes assigned Pfam domains	3,102	87.8	3,100	88.9
Genes with signal peptides	341	9.7	329	9.4
Genes with transmembrane helices	860	24.3	853	24.5
CRISPR repeats	0	0	1	0

sequence similarities, 97.1–97.5%) at the time of publication. Nonetheless, after the 16S rRNA gene sequence of *S. flexibilis*, ATCC 29606^T became available in the public sequence database in 2010, the closest relatedness between *P. tuomuerensis* 78-123^T and *S. flexibilis* ATCC 29606^T was finally discovered.

According to the literature, *S. flexibilis* and *P. tuomuerensis* exhibit a very similar cellular morphology with multiple flagella [11,33]. The high phenotypic similarities and sequence relatedness raised the strong possibility that the two species could be united into a single species. Thus, in order to clarify the taxonomic statuses of the two species, a polyphasic taxonomy study was conducted. The type strains of *S. flexibilis* ATCC 29606^T and *P. tuomuerensis* JCM 14085^T were obtained from the respective culture collections.

In order to investigate the genomic features, whole genome sequencing of *S. flexibilis* ATCC 29606^T and *P. tuomuerensis* JCM 14085^T was performed using the MiSeq system (Illumina) (Table 1). The paired reads were assembled using Genomics Workbench version 6.5.1 (CLC bio). Genes were predicted with Rapid Annotation using Subsystem Technology (RAST) server databases [4] and the gene-caller GLIMMER 3.02 [7]. The predicted open reading frames (ORFs) were annotated by searching clusters of orthologous groups (COGs) using the SEED databases [5] as well as NCBI databases. RNAmmer 1.2 [15] and tRNAscan-SE 1.23 [17] were used to identify rRNA genes and tRNA genes, respectively. CRISPR repeats were examined using CRISPR recognition tool (CRT) [6]. The sequencing reads of *S. flexibilis* were assembled into 49 contigs (>1-kb long) with an average coverage of 417×. The genome size of *S. flexibilis* was 3,762,876 bp with a G + C content of 65.79 mol%, which was in agreement with the value of 66 mol% reported by Hespell [11]. The genome of *P. tuomuerensis* was assembled into 16 contigs (>1-kb long) with an average coverage of 678×. The genome size of *P. tuomuerensis* was 3,748,402 bp with a G + C content of 65.83 mol%, which was significantly higher than the value of 60.4 mol% reported by Xin *et al.* [33]. The overall genomic contents of the two strains were very similar, containing 3,540 and 3,504 predicted coding sequences (CDSs), respectively. The genomic relatedness of the two test strains was calculated by average nucleotide identity (ANI) [14]. The ANI between the two strains was 98.1%, which is clearly above the suggested ANI value (95–96%) for demarcating genomic species [3,10,14,23]. In contrast, the ANI values ranged 76.7–77.2% when compared with other close relatives (*P. mendocina*, *P. pseudoalcaligenes*, and *P. stutzeri*) sharing >97% 16S rRNA gene sequence similarities with *S. flexibilis* (Table S1).

We also employed genome-sequence-based digital DNA-DNA hybridization (dDDH), which has been proposed to replace wet-lab DDH [3,18]. The dDDH value was calculated using the genome-to-genome distance calculator (GGDC) Version 2 by using identities/HSP length option (http://ggdc.dsmz.de). The dDDH value between the two strains was 89.0%, higher than the 70% DDH criterion for bacterial species affiliation [28,30]. *P. mendocina*, *P. pseudoalcaligenes*, and *P. stutzeri* yielded dDDH values 21.8–22.3% (Table S1), clearly demonstrating that those species are distinguished from *S. flexibilis* and *P. tuomuerensis*.

The two genome-derived 16S rRNA gene sequences (KP973996 and KP973997) differed at one nt position among the 1,530 bp (99.9% similarity). The obtained sequences were aligned with other reference sequences (showing >96% sequence similarity) using EzEditor [12], and phylogenetic analyses were performed using Mega 6.06 [27]. Evolutionary distance was calculated based on the Jukes & Cantor model [13], and phylogenetic trees were inferred based on the neighbor-joining [24] and maximum-likelihood [9] models. The tree topologies were evaluated by bootstrap analyses [8]. The close relatedness of the two species was also supported by the phylogenetic tree of the 16S rRNA gene sequences (Fig. 1). *S. flexibilis* ATCC 29606^T occupied a distinct lineage within the cluster enclosed by the genus *Pseudomonas* and formed a robust clade with *P. tuomuerensis* JCM 14085^T.

In addition to 16S rRNA gene sequence, the genome-derived sequences of three housekeeping genes, namely *gyrB*, *rpoB*, and *rpoD*, were analyzed together with reference sequences obtained from public databases. The sequence similarity values between *S. flexibilis* and *P. tuomuerensis* were 99.0–99.7%, while the values against the other close relatives were all lower than 90% (Table S1). The phylogenetic trees also confirmed the close relationship of *S. flexibilis* and *P. tuomuerensis* (Fig. S1).

The physiological and biochemical properties were examined using the API 20NE and API ZYM identification systems (bioMérieux) at 30 °C by following the manufacturer's recommendations. No striking differences in the phenotypic features were observed between the two strains. However, the two strains were distinguishable only by the assimilation of capric acid, for which strain ATCC 29606^T was positive whereas strain JCM 14085^T was negative. The results of the phenotypic and chemotaxonomic tests are presented in the species description and in Table S2. The fatty acid methyl esters (FAMEs) in whole cells were analyzed with GC (model 7890B; Agilent Technologies) according to the instructions of the Microbial Identification System (MIDI; version 6.2) with the TSBA6 database. The cells of the two type strains were grown on nutrient agar (Difco) at 30 °C for 24 h. Both strains showed similar fatty acid profiles (Table 2) with $C_{18:1}$ $\omega 6c$ and/or $C_{18:1}$ $\omega 7c$ (34.6–37.8%), $C_{16:1}$ $\omega 6c$ and/or $C_{16:1}$ $\omega 7c$ (14.5–20.2%), and $C_{16:0}$ (19.0–22.2%) constituting the major fatty acids (>10%).

On the basis of the high genomic relatedness (98.1% ANI value and 89.0% dDDH value), the high 16S rRNA gene sequence similarity (99.9%), and undistinguishable chemotaxonomic and phenotypic characteristics, it appears appropriate to combine *S. flexibilis* and

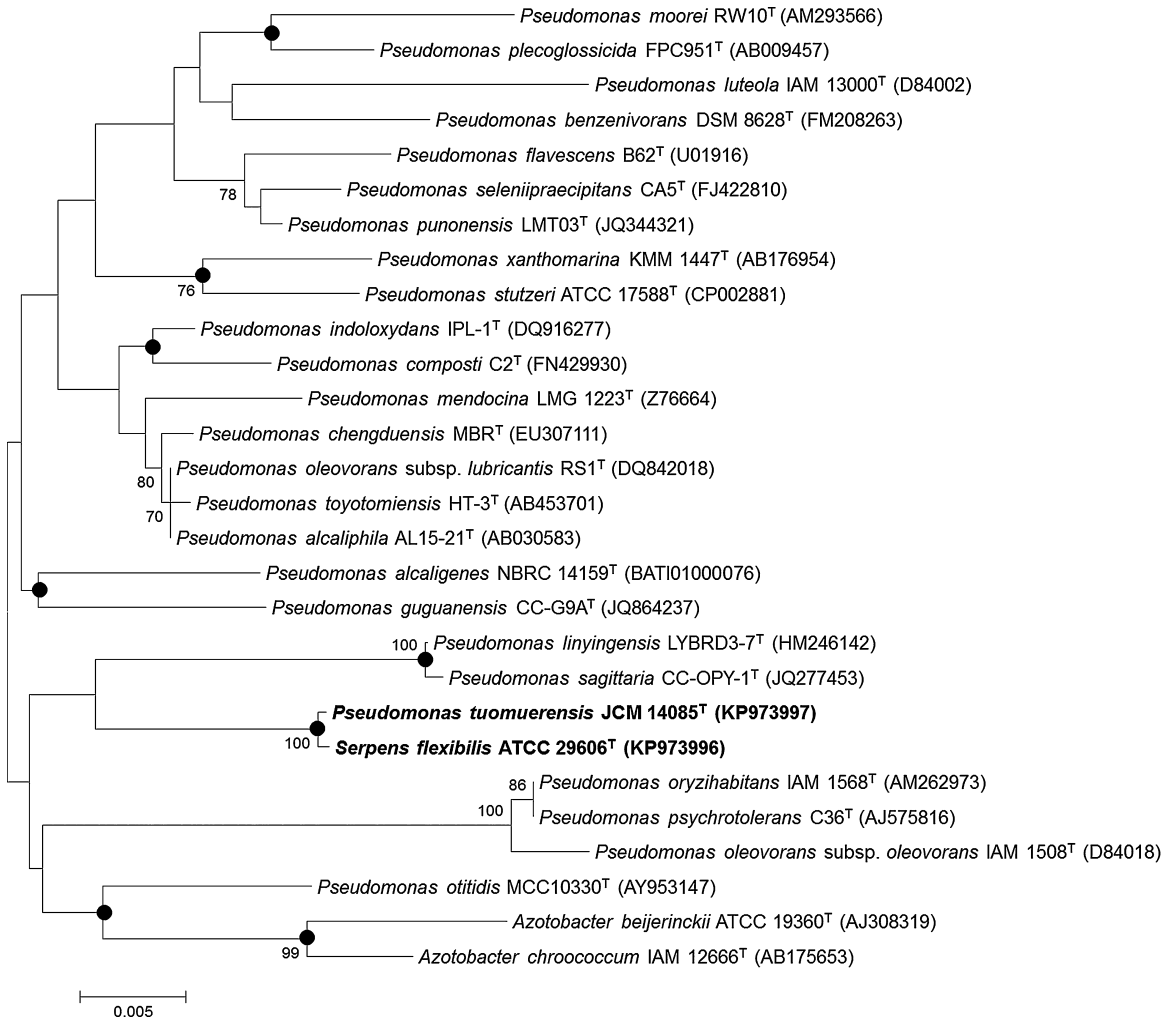

Fig. 1. Neighbor-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationship among *Serpens flexibilis*, *Pseudomonas tuomuerensis*, and other related species (>96% sequence similarities to *S. flexibilis*). The numbers at the nodes are given as percents and represent the levels of bootstrap support (>70%) based on neighbor-joining analyses of 1000 re-sampled data sets. The circles indicate that the corresponding nodes (groupings) were also recovered in the maximum-likelihood tree. Scale bar, 0.005 nt substitutions per position.

Table 2
Cellular fatty acid compositions of *Serpens flexibilis* ATCC 29606^T and *Pseudomonas tuomuerensis* JCM 14085^T.

Fatty acids	*S. flexibilis*	*P. tuomuerensis*
Saturated		
$C_{12:0}$	7.3	5.6
$C_{14:0}$	5.1	5.0
$C_{16:0}$	19.0	22.2
$C_{17:0}$	ND	Tr
$C_{18:0}$	Tr	7.4
Unsaturated		
$C_{17:1}$ $\omega 8c$	1.1	1.8
$C_{18:1}$ $\omega 9c$	ND	1.1
Hydroxy		
$C_{10:0}$ 3-OH	5.0	3.5
$C_{12:0}$ 3-OH	4.1	2.8
Summed feature*		
3	20.2	14.5
8	37.8	34.6

All data listed were obtained in this study. Values are percentages of total fatty acids. Fatty acids representing more than 0.5% are shown. ND, not detected; Tr, traces (<1%).
*Summed feature 3 comprises $C_{16:1}$ $\omega 6c$ and/or $C_{16:1}$ $\omega 7c$. Summed feature 8 comprises $C_{18:1}$ $\omega 6c$ and/or $C_{18:1}$ $\omega 7c$.

P. tuomuerensis into a single species. According to Rule 24 of the Bacteriological Code [16], we propose to transfer *Serpens flexibilis* Hespell 1977 to the genus *Pseudomonas* as *Pseudomonas flexibilis* comb. nov., with *Pseudomonas tuomuerensis* Xin *et al.* 2009 as a later heterotypic synonym of *P. flexibilis*.

Description of *Pseudomonas flexibilis* (Hespell 1977) comb. nov.

Pseudomonas flexibilis [fle.xi'bi.lis. L. fem. adj. *flexibilis* flexible, pliant].

Basonym: *Serpens flexibilis* Hespell 1977.

Heterotypic synonym: *Pseudomonas tuomuerensis* Xin *et al.* 2009.

The description is the same as that given by Hespell [11] and Xin *et al.* [33], with the following amendments. Cells are Gram-reaction-negative, aerobic, and motile with flagella. Colonies are white, round, and waved on the edge. The species grows at 15–42 °C, but not at 4 or 45 °C and is oxidase- and catalase-positive. It reduces nitrate; does not produce indole from tryptophan; does not hydrolyze gelatin; does not assimilate glucose, arabinose, mannose, mannitol, maltose, *N*-acetyl-glucosamine, trisodium citrate, potassium gluconate, or phenylacetic acid as

a sole carbon source; produces C4 esterase, C8 esterase lipase, leucine arylamidase, valine arylamidase, and naphthol-AS-BI-phosphohydrolase, but not urease, arginine dihydrolase, C14 lipase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, or α-fucosidase. Assimilation of capric acid varies depending on strains. The major cellular fatty acids are $C_{18:1}$ ω6*c* and/or $C_{18:1}$ ω7*c*, $C_{16:1}$ ω6*c* and/or $C_{16:1}$ ω7*c*, and $C_{16:0}$. The DNA G + C content is 65.8 mol%. The type strain is ATCC 29606^T. GenBank accession numbers for the 16S rRNA gene sequence and the whole genome sequence of the type strain are KP973996 and JRUD01000000, respectively.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.syapm.2015.09.007.

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