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Starkeya nomas sp. nov., a prosthecate and budding bacterium isolated from an immunocompromized patient

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Abstract

Strain HF14-78462^T is an environmental bacterium found in clinical samples from an immunocompromized patient in 2014 at Hospital Universitari i Politècnic La Fe (Valencia, Spain). Phenotypically, strain HF14-78462^T cells were Gram-stain-negative, aerobic, non-spore forming and non-motile small rods which formed mucous and whitish-translucent colonies when incubated at 20–36 °C. Phylogenetic analyses based on the 16S rRNA genes and the whole genomes of closest sequenced relatives confirmed that strain HF14-78462^T is affiliated with the genus *Starkeya*. The strain was oxidase, catalase and urease positive; but indole, lysine decarboxylase, ornithine decarboxylase and DNase negative, did not produce H_2S and was able to utilize a wide variety of carbon sources including acetamide, adonitol, amygdalin, L-arabinose, citric acid, glucose, mannitol and melibiose. Unlike *Starkeya novella* and *Starkeya koreensis*, strain HF14-78462^T failed to grow in thiosulphate-oxidizing media and had a narrower temperature growth range. Its genome was characterized by a size of 4.83 Mbp and a C+G content of 67.75mol%. Major fatty acids were $C_{18:1}$ $\omega 7c$, cyclo $C_{19:0}$ and $C_{16:0}$, its polar acids were diphosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol and an aminophospholipid; while the ubiquinones were Q9 (1.8%) and Q10 (98.2%). Digital DNA–DNA hybridization values were 41 and 41.4 against *S. novella* and *S. koreensis*, respectively, while average nucleotide identity values were around 84%. Phenotypic, average nucleotide identity and phylogenomic comparative studies suggest that strain HF14-78462^T is a new representative of the genus *Starkeya* and the name *Starkeya nomas* sp. nov. is proposed. The type strain is HF14-78462^T (=CECT 30124^T=LMG 31874^T).

INTRODUCTION

The genus *Starkeya* comprises two species: *Starkeya novella* and *Starkeya koreensis*, included in the family *Xanthobacteraceae* within the class *Alphaproteobacteria*. The first bacteria within this genus were isolated and described by R.L. Starkey in the early 1930s during an investigation of thiosulphate-utilizing bacteria [1]. These Gram-stain-negative bacteria were included in the genus *Thiobacillus* based on phenotypic characteristics, although they differed to a great extent from every other member of this genus because they could grow as autotrophs [1, 2]. *Thiobacillus* remained as a very heterogeneous genus until D.P. Kelly undertook the task of reclassifying its members by using 16S rRNA gene sequencing [3]. Thus, *Thiobacillus novellus* was reclassified as a new genus with only one species, and which name was chosen to honour its discoverer, i.e., *Starkeya novella*. In 2006, a new chemolithoautotrophic bacterium that was found in rice straw in Korea was added to this genus [4].

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Keywords: emerging pathogens; immunocompromized patient; *Starkeya*.
Abbreviations: ANI, average nucleotide identity; dDDH, digital DNA–DNA hybridization; ENA, European Nucleotide Archive.
Whole-genome shotgun project: PRJEB35169. Genome assembly and raw data: GCA_902703545 and ERR3672187. 16S rRNA gene sequence project: PRJEB40119; accession number: LR880920.

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A new, unclassified bacterium was first isolated in 2014 from several clinical samples of an immunocompromized patient at the Hospital Universitari i Politècnic La Fe, Valencia, Spain. Initial identification incorporated this isolate into the genus *Starkeya*, but phenotypic features as well as 16S rRNA analysis, average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH), identified this bacterium as representing a new species within the genus *Starkeya*. Thus, we propose strain HF14-78462^T as the type strain of a new species with the name *Starkeya nomas* sp. nov.

ISOLATION AND ECOLOGY

Starkeya are environmental Gram-stain-negative bacteria, and the two known species, *S. novella* and *S. koreensis*, have been found in soil and rice straw, respectively [1, 4].

In 2014, a 33-year-old woman complaining of 48 h of fever, nasal congestion, bilateral otalgia and sputum producing-cough presented to the emergency room of the Hospital Universitari i Politècnic La Fe. She had been diagnosed with Hodgkin's disease 4 years earlier and had received allogeneic haematopoietic progenitor cell transplantation from a sister a year before. After transplantation, she suffered a multidrug resistant-*Pseudomonas aeruginosa* bacteraemia, pneumonia by *P. aeruginosa*, *Aspergillus terreus* and *Parainfluenza* virus type I, and Epstein-Barr virus and Cytomegalovirus reactivation. She was taking 20 mg day^{-1} prednisone for the treatment of chronic graft-*versus*-host disease, and on prophylaxis with acyclovir, co-trimoxazole and voriconazole.

Physical examination showed a temperature of 35.6 °C, a heart rate of 128 b.p.m., a blood pressure of 113/61 mmHg, and a 95% O_2 saturation. Laboratory tests did not reveal any abnormalities except for leucopenia (2.3×10^9 leukocytes/l), anaemia (red cells: 2.79×10^{12} /l; haemoglobin: 8.9 g dl^{-1}), thrombocytopenia (platelets: 26×10^9/l), urea (70 mg dl^{-1}), creatinine (1.3 mg dl^{-1}), aspartate aminotransferase (255 U l^{-1}), fibrinogen (599 mg dl^{-1}) and C-reactive protein (79 mEq l^{-1}). A chest X-ray showed cicatricial atelectasis at the upper left lobe, but no alveolar consolidation, pulmonary nodules or pleural effusion. The patient carried a right subclavian central venous catheter with end in the right atrium (tunnelled Hickman) for a year which was maintained during admission. Six days before admission, a bone marrow puncture had been performed for cytological and microbiological control.

A non-fermenting, immobile, Gram-negative rod was isolated in the bone marrow after 8 days of incubation in BACTEC MYCO/F Lytic bottle (BD). Subcultures on blood agar incubated at 36 °C grew mucous and translucent colonies, but failed to grow at 4 or 42 °C nor in the presence of NaCl 6.5%. An aerobic blood culture (BacT-ALERT, bioMérieux) drawn from the Hickman catheter during her hospital stay yielded the same bacterium after 3 days of incubation. The isolate could not be identified by conventional automated systems (Vitek2 GN IDcard, bioMérieux and BBL Crystal E/NF, BD) nor by MALDI-TOF (Vitek MS, bioMérieux; and Bruker Biotyper, Bruker Daltonics).

Antimicrobial susceptibility was performed on Mueller-Hinton-Fastidious agar (Becton Dickinson) by E-test according to the manufacturer's recommendations. Minimum inhibitory concentrations in μg ml^{-1} were: ampicilin 1.5, cefoxitin 0.38, cefotaxime 0.38, co-trimoxazol 0.5, amikacin 0.38, gentamicin 0.023, tobramycin 0.023, imipenem <0.002, meropenem <0.002, aztreonam 24, levofloxacin 1 and ciprofloxacin 2.

After 11 days on intravenous meropenem together with amikacin the first five, the patient was discharged with the diagnosis of upper respiratory tract infection and otitis media, and was placed for 5 more days on levofloxacin 500 mg every 24 h. Two months later, the patient presented to the outpatient clinic complaining of malaise, 2–3 loose stools per day, nausea and vomiting. Laboratory tests showed anaemia (red cells: 2.47×10^{12}/l; haemoglobin: 8.5 g dl^{-1}) and thrombocytopenia (platelets: 12×10^9/l). A stool culture grew *Campylobacter jejuni*; and a Hickman catheter-drawn blood culture grew *Starkeya* species after 48 h; antimicrobial susceptibility being the same. Treatment was started with ciprofloxacin 500 mg every 12 h and the Hickman catheter was removed. Subsequent blood cultures were negative.

Thus, given the immune status of the patient and the nature of this bacterium, it is very likely that the patient was infected and colonized from an environmental source. Furthermore, a similar genome from a soil bacterium isolated in Russia has been recently published in the ENA (European Nucleotide Archive) database, *Starkeya* sp. 3C (genome assembly accession number ASM755943v1) with a 98.62% ANI, and an 82.8% dDDH identity (Fig. 1, Table 1) with our isolate, which would mean that it belongs to the same species as strain HF14-78462^T.

Starkeya has not been previously described as a human pathogen; therefore, nothing is known about its clinical significance, virulence or antimicrobial susceptibility. In this case, the entry port was most likely the Hickman catheter, since strain HF14-78462^T was isolated from catheter drawn-blood cultures; and after its removal, subsequent blood cultures were negative. Unfortunately, the removed Hickman catheter could not be properly studied.

In this immunocompromized patient, the first isolation occurred in a routinely haematological study, the second in blood culture collected during an infection of the upper respiratory tract, and the third in a diarrhoea episode caused by *C. jejuni*. Altogether, these associations seem to indicate little pathogenic potential.

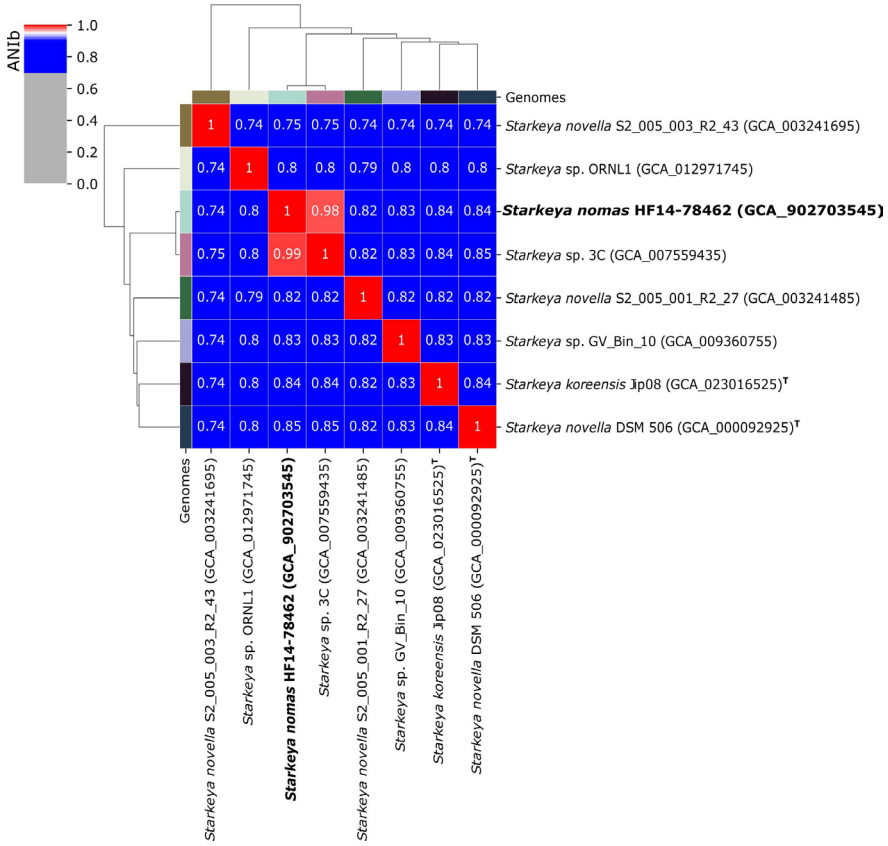

Fig. 1. Heatmap output of the pyani software, with the average nucleotide identity percentages depicted for each genomic comparison, with row and column dendrograms of similarity clustering.

All in all, our data points to a free-living bacterium that, opportunistically, infected a human being. The fact that this bacterium has been isolated in geographically separated locations, such as Russia and Spain, could mean that it is widely distributed.

16S rRNA GENE PHYLOGENY

Phylogenetic reconstruction of the 16S rRNA gene (Fig. 2) was performed with the software RaxML version 8 [5] with 10 000 bootstrap replicas, with selected sequences of the genus *Starkeya* and those of the type strains of the family *Xanthobacteraceae* available from the SILVA database [6]. These sequences had to be at least 1400 bp long, with more than 90% of high sequence quality to be considered in our analysis. The software MAFFT [7] was used to align the sequences, and the software JModelTest

Table 1. Digital DNA–DNA hybridization percentages between the Starkeya genomes available in NCBI database and the strain presented in this work. [T] refers to type trains

	Strain and acc. no.	1	2	3	4	5	6	7	8
1	Starkeya koreensis Jip08 [T] GCA_023016525	*							
2	Starkeya novellaS2_005_001_R2_27 GCA_003241485	45.4	*						
3	Starkeya novella DSM 506 [T] GCA_000092925	45.4	100	*					
4	Starkeya sp. HF14-78462 [T] GCA_902703545	41.4	41	41	*				
5	Starkeya sp. 3C GCA_007559435	47	44.8	44.8	82.8	*			
6	Starkeya novella S2_005_003_R2_43 GCA_003241695	35.3	33.8	33.8	33.3	35.4	*		
7	Starkeya sp. GV_Bin_10 GCA_009360755	30.9	27	27	24.9	26.7	24.9	*	
8	Starkeya sp. ORNL1 GCA_012971745	23.6	25.3	25.3	21.7	22.5	21.7	18.4	*

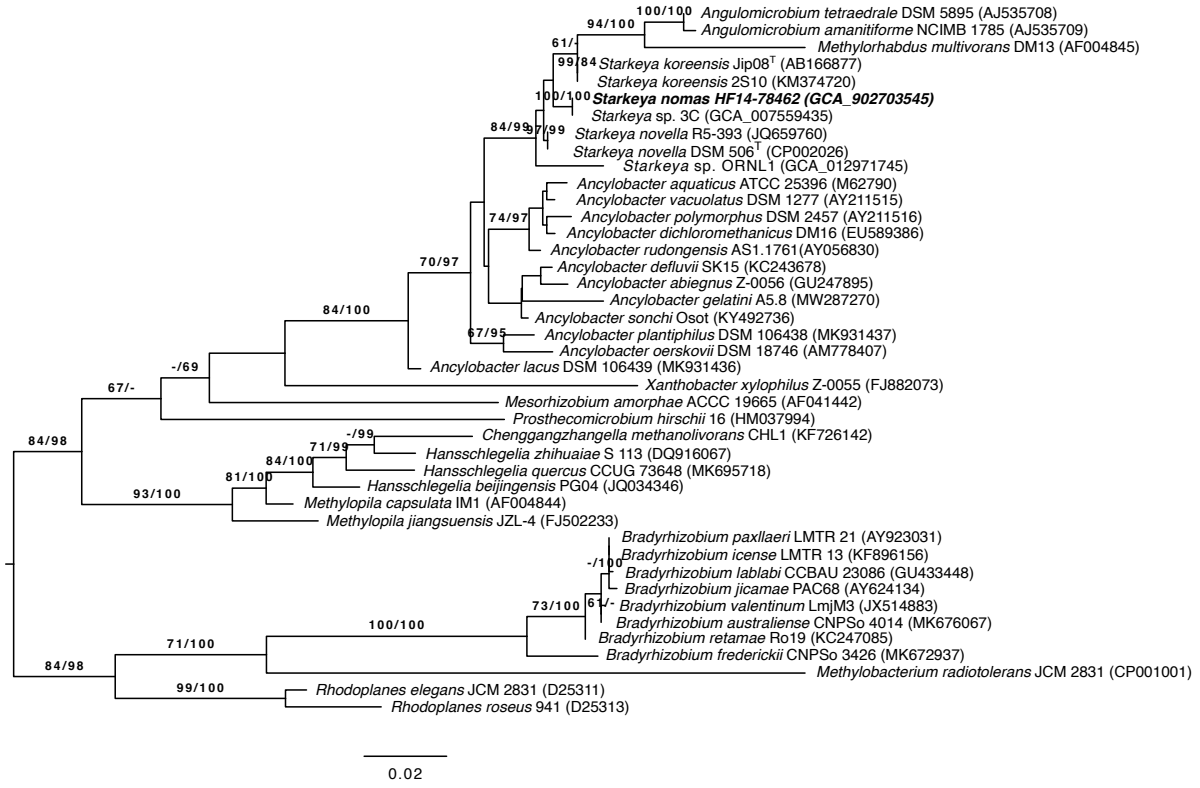

Fig. 2. 16S rRNA phylogenetic reconstruction of the type strains of *Xanthobacteraceae* and *Starkeya* sp. sequences available in the SILVA database.

[8] to infer the best model for the phylogenetic reconstruction. Model GTR+I+64 was the best to reconstruct this phylogenetic tree, with a rejection of sequences below identity of 80%.

GENOME FEATURES

Genomic DNA isolation from this bacterium was carried out using a column method, with the prep kit QiAmp DNA stool Minikit with reference 51504 and customized parameters. The DNA concentration measurements were quantified with Qubit High Sensitivity, and genomic DNA was sequenced using 250 bp paired-end reads on an Illumina MiSeq platform by means of a Nextera XT library prep kit. Sequencing resulted in a total of 20249672 reads, with a median insert size of 266 bp.

To assess the quality control of the reads, we used the software Prinseq-lite [9] with the following parameters: longitud_min:50, trim_qual_right:30, trim_qual_type:mean and trim_qual_window:20. A *de novo* assembly was performed with SPAdes version 3.13.0 [10]. SSPACE and Gapfiller [11, 12] were used to construct the scaffold of the genome, and to close gaps and nucleotides, respectively. MeDuSa [13] was also employed for further scaffolding based on a reference, where we used the assembly of *Starkeya* sp. 3C, and the software BUSCO [14] provided quantitative measures for the assessment of genome assembly.

From the assembled contigs, we searched for the orthologous groups in BUSCO, a program that assesses the completeness of a genome assembly by searching for 40 core genes in OrthoDB version 9. We found 39 out of the 40 orthologues searched for as complete and one partial. The final draft of strain HF14-78462^T has 53 contigs, with a mean coverage of 81×. The N50 value is 251015 bp and the largest contig has 469523 bp. The genome size is 4833713 bp with a C+G content of 67.75 mol%. Furthermore, 4542 CDSs, five rRNAs, 51 tRNAs and one tmRNA gene were predicted. Lastly, the draft genome was annotated with the software Prokka [15] and translated to an embl file with the software GFF3toEMBL [16].

Additionally, we calculated the ANI and the dDDH values between strain HF17-78462^T and six *Starkeya* genome assemblies/drafts available in NCBI with the software pyani [17], and the Type Strain Genome Server (TYGS) [18], respectively (Fig. 1, Table 1). The dDDH value is measured with the formula d_0, that measures the length of all the high scoring pairs divided by total genome length. The result for both strain HF14-78462^T and *Starkeya* sp. 3C was 'potential new species'.

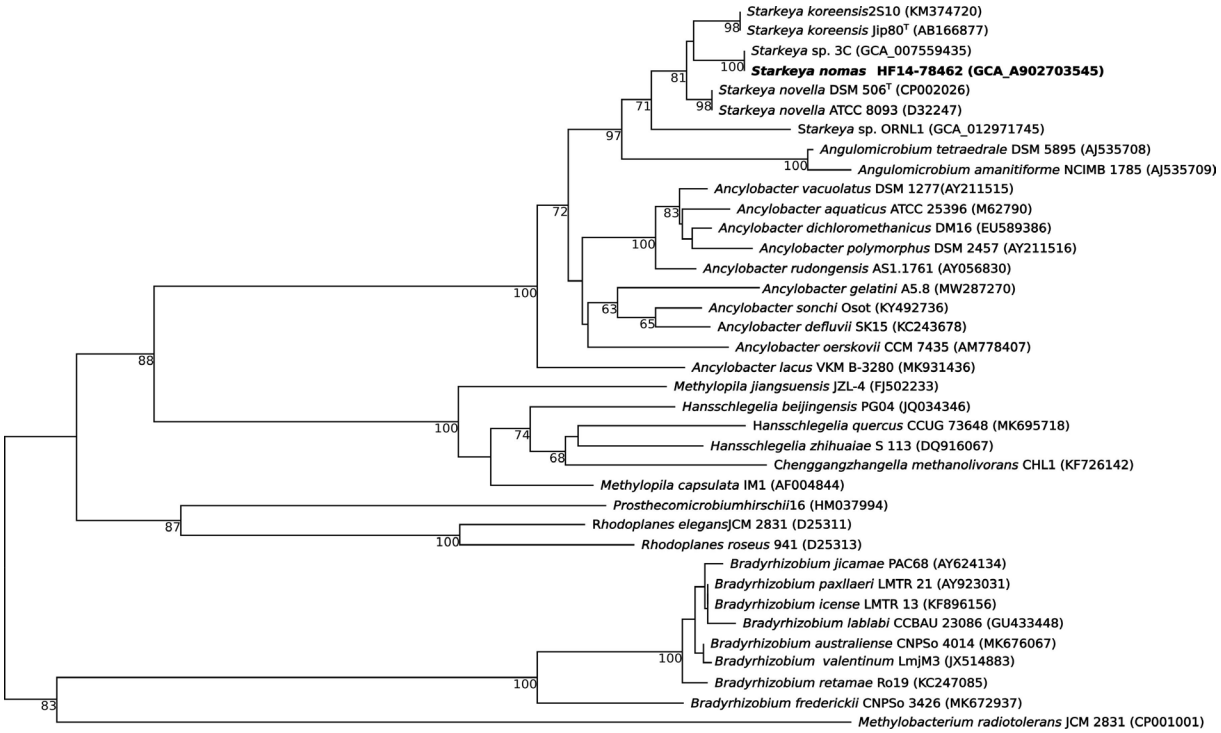

Fig. 3. 16S rRNA phylogenetic reconstruction of *Starkeya* species studied in this work and their closest relatives in the TYGS web server.

Furthermore, the resulting intergenomic distances of the organisms in Table 1 were used to infer two additional balanced minimum evolution genomic trees with branch support with FastME 2.1.6.1 including SPR postprocessing [19] within the web server TYGS: a 16S rRNA gene phylogenetic reconstruction from genome BLAST distance phylogeny (GBDP) distances calculated from 16S rRNA gene sequences. The branch lengths are scaled in terms of GBDP distance formula d_5. The numbers above branches are GBDP pseudo-bootstrap support values >60% from 100 replications, with an average branch support of 70.0% (Fig. 3) and a whole-genome phylogenetic reconstruction from GBDP distances calculated from genome sequences. The branch lengths are scaled in terms of GBDP distance formula d_5. The numbers above branches are GBDP pseudo-bootstrap support values >60% from 100 replications, with an average branch support of 89.3% (Fig. 4). Branch support was inferred from 100 pseudo-bootstrap replicates each, and the trees were rooted at the midpoint. With information from the 16S rRNA and the genomic phylogenetic reconstructions, the ANI and the dDDH values resulting of our analysis, we conclude that *Starkeya* sp. 3C and *Starkeya* sp. HF14-78462^T belong to the same species, and we propose the name *Starkeya nomas* sp. nov. for them. Moreover, we are also able to conclude that the strain *Starkeya* sp. ORNL1 is wrongfully taxonomically assigned, and it should not be included in the genus *Starkeya*.

PHYSIOLOGY AND CHEMOTAXONOMY

Cells of HF14-78462^T were Gram-stain-negative, aerobic, non-spore forming and non-motile small rods occurring singly. Prosthecae and budding was observed in accordance with members of the family *Xanthobacteraceae* [20] (Fig. 5). Aerobic cultures on Columbia blood sheep agar incubated at 36 °C grew mucous and whitish-translucent colonies. *Starkeya* HF14-78462^T failed to grow in 6.5 and 10% NaCl containing media but was able to grow on thioglycollate supplemented with up to 5% NaCl. The bacterium grew at a temperature of 20 and 36 °C, but not at 4, 8, 42 or 56 °C.

Chemolithoautotrophic growth on thiosulphate was tested in a thiosulphate-oxidizing medium as described in Atlas [21] which was provided by the Unidad Analítica, IIS La Fe. No growth was observed after 10 days of incubation.

Oxidase activity was determined with BD Oxidase Reagent Dropper 0.5 ml of a 1% aqueous solution of *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (Beckton Dickinson) by streaking a loopful of bacteria onto a reagent-saturated filter paper. Oxidase test was considered positive if the colour of the filter paper turned dark purple. For the catalase activity, a loopful of bacteria was immersed into a tube with 3% H_2O_2. The presence of bubbles indicated if the test was positive.

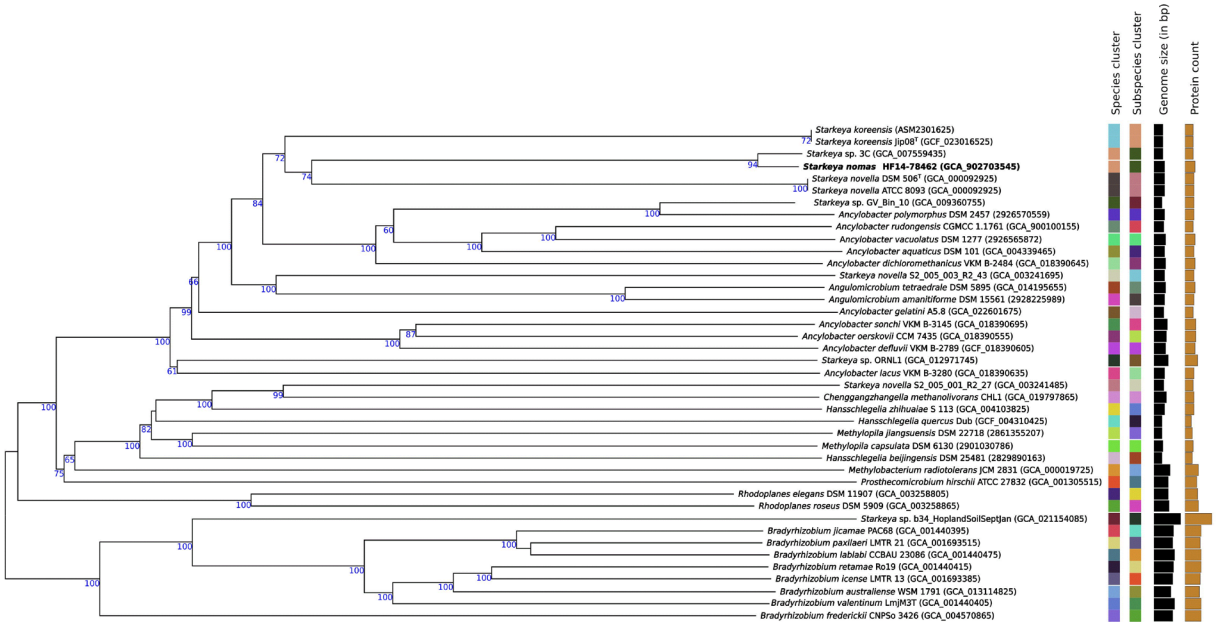

Fig. 4. Whole genome reconstruction of the *Starkeya* species genomes studied in this works and their closest relatives included in the TYGS web server.

DNase production was tested on DNase agar plates (Maim S.L.) by streaking a thick line of inoculum of strain HF14-78462^T across the plate. Plates were incubated at 35–37 °C for 10 days in a humidity test chamber, after which period, the agar plates were flooded with 1 N hydrochloric acid to reveal if there are any clear or transparent zones surrounding the streak. If the plate becomes totally turbid without any clear zone, then the test is negative; however, if any clear zones develop around the growth, the test is described as positive. Strain HF14-78462^T was DNase negative, but oxidase and catalase positive.

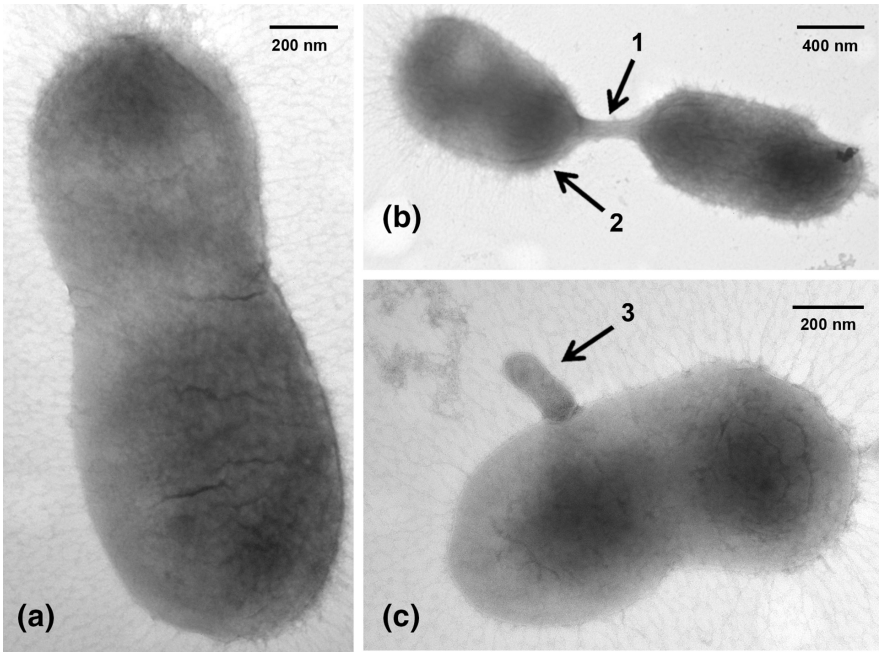

Fig. 5. Negative staining electron microscopy of strain HF14-78462^T. Normal morphology (a), polar prostheca (b1), budding (b2) and lateral prostheca (c3).

Table 2. Differential phenotypic characteristics of strain HF14-78462^T and species from the genus *Starkeya*

Strains: 1, HF14-78462^T; 2, *S. koreensis* DSM 18406^T; 3, *S. novella* DSM 506^T. Data from this study. +, Positive; –, negative; v, different results were obtained from different kits.

Characteristics	1	2	3
Cell morphology:			
Shape	Short rod	Highly curved rod	Short rod
Length (μm)	0.8–2.0	0.8–2.0	0.8–2.0
Width (μm)	0.4–0.8	0.4–0.8	0.4–0.8
Fatty acids	$C_{18:1}$ ω7*c*, cyclo $C_{19:0}$, $C_{16:0}$	$C_{18:1}$ ω7*c*, cyclo $C_{19:0}$, $C_{16:0}$, $C_{18:0}$	$C_{18:1}$ ω7*c*, cyclo $C_{19:0}$*c*
Physiology and growth:			
Temperature range (°C)	20–36	4–40	8–37
Growth on thiosulphate medium	–	+	+
Enzyme production:			
Arginine dihydrolase	v	–	–
Urease	+	+	–
Lysine decarboxylase	–	+	–
Hydrolysis of:			
p-n-p-N-Acetyl-glucosaminide	–	+	+
Aesculin	+	–	–
p-n-p-β-Galactoside (ONPG)	+	+	–
Gelatin	+	+	–
γ-L-Glutamyl	–	–	+
L-Proline	–	+	+
L-Tryptophan	–	+	+
Lysine-alanine	–	+	+
Utilization of:			
Acetamide	+	–	–
N-Acetylglucosamine	–	–	+ (–)*
Adonitol	+	–	–
Amygdalin	+	–	–
Citric acid	+	+	–
D-Sorbitol	–	–	+
Mannitol	+	–	+
Melibiose	+	–	–
L-Rhamnose	v	–	+
Malonic acid	v	–	+ (–)*
Gluconic acid	–	–	– (+)*

*Different results reported by Im *et al.* [4] (in parentheses).

S. novella DSM 506^T and *S. koreensis* DSM 18406^T were obtained from the Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, for comparing their biochemical profiles with strain HF14-78462^T. Biochemical tests were assessed with in-house and commercial kits –API 20E and API 20NE (bioMérieux), BD Phoenix NMIC/ID Panels (Beckton Dickinson) and MicroScan Gram-Negative MIC/Combo Panel (Beckman Coulter) – incubated at 36 °C and according to the manufacturers' instructions, but extending the incubation period for 10 days in a humidity test chamber. Differential biochemical results for the three *Starkeya* tested can be found in Table 2, and a detailed list is also provided in the description of strain HF14-78462^T. It is worth mentioning that some of the results obtained in our biochemical testing of

S. novella DSM 506^T and *S. koreensis* DSM 18406^T were different from those reported by Im *et al.* [4] as indicated in Table 2. Also, although urease production was positive for both the novel isolate and *S. koreensis* DSM 18406^T, the latter was weaker and slower.

Lipidome was extracted and saponified as described previously [22] followed by a LC-MS analysis of the total fatty acid on bacteria grown on sheep blood agar at 36 °C for 5 days. Chromatographic analysis was performed on a Thermo Scientific Vanquish UHPLC system equipped with an Acquity UPLC BEH C18 column (100×2.1 mm; 1.7 µm; Waters) and mass spectrometry analysis was carried out by an Q-exactive mass spectrometer from Thermo Scientific equipped with an electrospray source. Data analysis was performed using EI-Maven version 0.9.1 [23]. The analysis of the whole-cell fatty acid composition was very similar to the other *Starkeya* species [4] with $C_{18:1}$ *ω7c* (69%), cyclo-$C_{19:0}$ (22%) and $C_{16:0}$ (6%) as the major components.

Analysis of polar lipids and ubiquinones were carried out by DSMZ services, Leibniz-Intitute DSMZ- Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. The polar acids found were diphosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol and aminophospholipid; while the ubiquinones detected were Q9 (1.8%) and Q10 (98.2%).

Strikingly, there is an interesting feature that singles out strain HF14-78462^T from the other two members of *Starkeya*, i.e. its temperature growth range. While *S. koreensis* has the widest range of them all, strain HF14-78462^T has the narrowest. Moreover, strain HF14-78462^T presents a temperature growth range that seems adapted to human body temperature. Finding out if this is a common trait of the new species or just an adaptation of this strain is yet to be studied.

Considering the results of phylogenetic analysis and dDDH and ANI relatedness studies and, based on phenotypic evidence, we propose that strain HF14-78462^T represents a novel species in the genus *Starkeya*, for which the name *Starkeya nomas* sp. nov. is proposed.

DESCRIPTION OF *STARKEYA NOMAS* SP. NOV.

Starkeya nomas (no'mas. L. fem. n. *nomas*, wanderer, because it is an environmental bacterium found in Spain and Russia, but also within a human being).

Cells are Gram-stain-negative, aerobic, non-spore forming and non-motile small rods about 2 µm long and 0.7 µm wide occurring singly. Prosthecae and budding is observed. Growth occurs optimally between 20–36 °C and colonies are mucous and whitish-translucent on Columbia blood agar incubated at 36 °C. The strain is oxidase, catalase and urease positive; but indole, lysine decarboxylase, ornithine decarboxylase and DNase negative, does not produce H_2S, fails to grow on thiosulphate-oxidizing media and nitrate reduction to nitrite is negative.

Hydrolysis of aesculin, *p-n-p*-β-galactoside (ONPG), gelatin, *p-n-p* α-β-glucoside, arginine-arginine, glycine, glutaryl-glycine-arginine, L-arginine, L-leucine and L-proline is positive, whereas it is negative for *p-n-p-N*-acetyl-glucosaminide, L-glutamic acid, *p-n-p* bis-phosphatel-pyroglutamic acid, γ-L-glutamyl, L-proline, L-tryptophan and lysine-alanine.

The bacterium is able to utilize a wide variety of carbon sources including acetamide, adonitol, amygdalin, L-arabinose, citric acid, glucose, mannitol and melibiose; but not *N*-acetyl-glucosamine; sorbitol, galactose, inositol, raffinose, sucrose, acetate, α-ketoglutaric acid, tiglic acid, β-allose, β-gentibiose, dextrose, fructose, galacturonic acid, methyl β-glucoside, maltulose, *N*-acetyl-galactosamine, sucrose, malonic acid or gluconic acid.

Its genome is characterized by a size of 4.83 Mbp and a C+G content of 67.75mol%. Major fatty acids are $C_{18:1}$ *ω7c*, cyclo-$C_{19:0}$ and $C_{16:0}$. The polar acids are diphosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol and an aminophospholipid; while the ubiquinones are Q9 (1.8%) and Q10 (98.2%).

The type strain is HF14-78462^T (=CECT 30124^T=LMG 31874^T), which was isolated from blood and bone marrow from an immunocompromized patient.

The whole-genome shotgun project of the type strain is in the ENA database with id: PRJEB35169. Genome assembly and raw data with ids GCA_902703545 and ERR3672187, respectively. 16S rRNA gene sequence project: PRJEB40119. Accession number: ERP123722

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Author contributions
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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
This work was approved by the ethics committee of the Hospital Universitari i Politècnic La Fe.

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