Casimicrobium huifangae gen. nov., sp. nov., a Ubiquitous "Most-Wanted" Core Bacterial Taxon from Municipal Wastewater Treatment Plants

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ABSTRACT Microorganisms in wastewater treatment plants (WWTPs) play a key role in the removal of pollutants from municipal and industrial wastewaters. A recent study estimated that activated sludge from global municipal WWTPs harbors 1×10^9 to 2×10^9 microbial species, the majority of which have not yet been cultivated, and 28 core taxa were identified as "most-wanted" ones (L. Wu, D. Ning, B. Zhang, Y. Li, et al., Nat Microbiol 4:1183–1195, 2019, https://doi.org/10.1038/s41564 -019-0426-5). Cultivation and characterization of the "most-wanted" core bacteria are critical to understand their genetic, physiological, phylogenetic, and ecological traits, as well as to improve the performance of WWTPs. In this study, we isolated a bacterial strain, designated SJ-1, that represents a novel cluster within *Betaproteobacteria* and corresponds to OTU_16 within the 28 core taxa in the "most-wanted" list. Strain SJ-1 was identified and nominated as *Casimicrobium huifangae* gen. nov., sp. nov., of a novel family, *Casimicrobiaceae*. *C. huifangae* is ubiquitously distributed and is metabolically versatile. In addition to mineralizing various carbon sources (including carbohydrates, aromatic compounds, and short-chain fatty acids), *C. huifangae* is capable of nitrate reduction and phosphorus accumulation. The population of *C. huifangae* accounted for more than 1% of the bacterial population of the activated sludge microbiome from the Qinghe WWTP, which showed seasonal dynamic changes. Cooccurrence analysis suggested that *C. huifangae* was an important module hub in the bacterial network of Qinghe WWTP.

IMPORTANCE The activated sludge process is the most widely applied biotechnology and is one of the best ecosystems to address microbial ecological principles. Yet, the cultivation of core bacteria and the exploration of their physiology and ecology are limited. In this study, the core and novel bacterial taxon *C. huifangae* was cultivated and characterized. This study revealed that *C. huifangae* functioned as an important module hub in the activated sludge microbiome, and it potentially plays an important role in municipal wastewater treatment plants.

KEYWORDS *Casimicrobium huifangae*, activated sludge microbiome, core taxa, microbial network, municipal wastewater treatment plant, WWTP, nitrogen and phosphorus removal

Microorganisms are the main players driving the removal of carbon, nitrogen, phosphorus, and micropollutants, such as antibiotics, endocrine-disrupting chemicals, and personal care products, from wastewaters. These microorganisms occur as biofilms, flocs, and aggregates in wastewater treatment plants (WWTPs), and they assemble in diverse patterns (1). The activated sludge process has been widely used in municipal WWTPs (2, 3) since its establishment in 1914 (4). The performance and removal efficiency of WWTP pollutants depend on the quality of the activated sludge, which in turn relies upon the activities of diverse and versatile microbial communities therein. Abnormal growth and dysbiosis of microbial communities in WWTPs resulted in low performance, such as the decreased removal efficiency of pollutants, bulking, or foaming of activated sludge (5–7).

The activated sludge microbiome consists of bacteria (1×10^{12} to 1×10^{13} cells/g of volatile suspended solids [VSS]), as well as archaea, viruses (bacteriophages), algae, protozoa, and metazoa (8, 9). Previous investigations have shown that members of *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Firmicutes* are predominant in activated sludge microbiomes (10, 11). *Proteobacteria* and *Bacteroidetes* accounted for more than half of the total bacterial sequences, and the abundance of *Acidobacteria*, *Actinobacteria*, *Firmicutes*, *Chloroflexi*, *Planctomycetes*, and *Verrucomicrobia* accounted for more than 1% (12–14). These bacteria are essential for ammonia and nitrite oxidization (e.g., *Nitrosomonas*, *Nitrospira*, and *Nitrotoga*), denitrification (e.g., *Thauera* and *Dechloromonas*), and glycogen and phosphorus accumulation and removal (e.g., *Defluviicoccus*, *Accumulibacter*, and *Tetrasphaera*), or they play a crucial role in the assembly and structuration of activated sludge flocs (such as *Zoogloea*, *Thiothrix*, *Chloroflexi*, and *Microthrix parvicella*) (15).

Although the activated sludge process has been used for more than 100 years and continuous efforts have been made toward dissection of the microbial community composition and structure, the current understanding of activated sludge microbiomes is very limited. An earlier review commented that only 1 to 15% of microbes had been cultivated from activated sludge (16). According to a recent estimation of bacterial diversity in global WWTPs, there are 1×10^9 to 2×10^9 microbial species, and 28 core taxa of functional importance have been identified and listed as the "most-wanted" activated sludge microbes (3). Considering that fewer than 10^5 microbial species have been cultivated and described, there are about 99.99% microbial species that have not yet been cultivated. Thus, the cultivation of key microbial taxa is greatly needed for further genetic, biochemical, physiological, ecological, and evolutionary research, as well as process engineering (1, 17).

Core microbial operational taxonomic units (OTUs) in global activated sludges have been identified by the Global Water Microbiome Consortium (GWMC) (http://gwmc.ou .edu/), but the understanding of their physiology and roles in WWTPs is very limited (3). We noticed that more than half of the 28 core taxa identified by the GWMC were annotated at the genus or family level. However, OTU_16 of *Betaproteobacteria* (3) was not able to be annotated to any currently known taxa. In this study, we worked extensively on core microbiome isolation and cultivation from activated sludge. In total, 830 bacterial isolates were obtained, and a strain, namely SJ-1, that corresponds to OTU_16 was cultivated and its genome was sequenced. Strain SJ-1 was characterized and identified as a new species, *Casimicrobium huifangae*, of the new family *Casimicrobiaceae*. Based on our results, we concluded that *C. huifangae* is ubiquitously distributed and potentially plays important roles in the removal of nutrient elements and networking with other microbes in activated sludge of WWTPs.

RESULTS AND DISCUSSION

Growth and characterization of *C. huifangae*. A total of 830 bacterial isolates were obtained; of these, 56.6% belonged to *Proteobacteria*, 25.1% to *Actinobacteria*, 13.3% to *Bacteroidetes*, 4.8% to *Firmicutes*, 0.5% to *Deinococcus-Thermus*, and 0.1% to *Verrucomicrobiae*. Based on analysis of full-length 16S rRNA gene similarity (cutoff values of <98%), 23.1% isolates represented previously uncultured and potentially novel taxa.
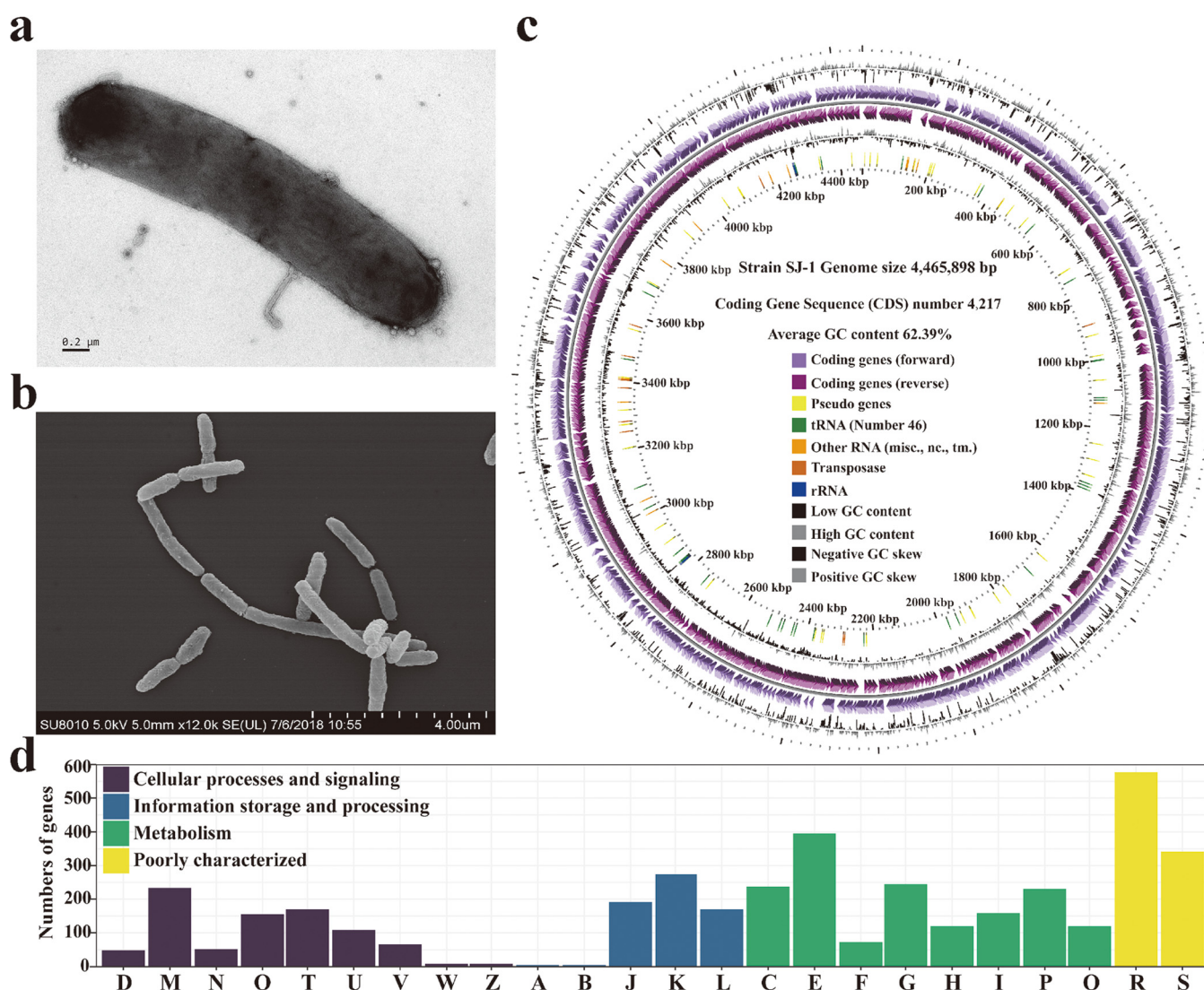
FIG 1 Morphology and genome of *C. huifangae* strain SJ-1. (a and b) Photos showing the cell morphology of strain SJ-1 under transmission (a) and scanning (b) electron microscopy. (c) A circular map of the chromosome of strain SJ-1. From the outside to the center, circle 1 illustrates the gene GC percent deviation (gene GC% − genome mean GC%), circle 2 illustrates the predicted CDSs transcribed in the clockwise direction, circle 3 illustrates the predicted CDSs transcribed in the counterclockwise direction, circle 4 illustrates the gene GC skew, and circle 5 illustrates rRNA (blue), tRNA (green), misc_RNA (orange), transposable elements (chocolate brown), and pseudogenes (yellow). (d) Numbers of genes of strain SJ-1 associated with general COG functional categories. Class identification includes cell cycle control, cell division, and chromosome partitioning (D), cell wall/membrane/envelope biogenesis (M), cell motility (N), posttranslational modification, protein turnover, and chaperones (O), signal transduction mechanisms (T), intracellular trafficking, secretion, and vesicular transport (U), defense mechanisms (V), extracellular structures (W), cytoskeleton (Z), RNA processing and modification (A), chromatin structure and dynamics (B), translation, ribosomal structure, and biogenesis (J), transcription (K), replication, recombination, and repair (L), energy production and conversion (C), amino acid transport and metabolism (E), nucleotide transport and metabolism (F), carbohydrate transport and metabolism (G), coenzyme transport and metabolism (H), lipid transport and metabolism (I), inorganic ion transport and metabolism (P), secondary metabolite biosynthesis, transport, and catabolism (Q), general function prediction only (R), and function unknown (S).

One isolate, named SJ-1, that represents a novel cluster of bacteria within *Betaproteobacteria* was elaborately investigated in detail. Strain SJ-1 was first isolated with 10-fold-diluted R2A agar, after extended cultivation for 3 weeks. It also grew on undiluted R2A agar and weakly on nutrient agar (NA) but did not grow on Trypticase soy agar (TSA) or LB agar. Colonies of strain SJ-1 on R2A agar were circular, convex, and smooth, white and semitransparent, with a wet surface. Colony diameters were between 0.5 and 0.8 mm after 3 days of incubation. When cultivated in broth, the organism needed a large inoculum (>1%, optical density at 600 nm [OD_{600}] ≥ 0.3) to secure its growth. The morphology of strain SJ-1 is shown in Fig. 1a and b. Cells were rods, 0.7 to 0.8 μm wide and 1.5 to 4.0 μm long. No flagella or pili were observed. Cells

were nonmotile and stained Gram negative. The organism did not form spores, did not grow under anaerobic conditions, and had a long lag phase for growth in R2A broth. The doubling time (or generation time) was 5.3 to 6.2 h under optimal conditions. Strain SJ-1 grew at 4 to 37°C, with optimum growth at 30 to 37°C. Strain SJ-1 grew at a pH range of 5.5 to 9.4 (optimum, pH 7.0 to 7.5) and at an NaCl concentration range of 0 to 0.5% (optimum, no NaCl addition). Strain SJ-1 hydrolyzed esculin, gelatin, casein, and Tween 20 but did not hydrolyze tyrosine, cellulose, starch, and Tween 40, Tween 60, or Tween 80. Enzyme activity tests were positive for oxidase, catalase, alkaline phosphatase, acid phosphatase, esterase (C_4), esterase lipase (C_8), leucine arylamidase, valine arylamidase, cystine arylamidase, naphthol-AS-BI-phosphohydrolase, trypsin, and α-chymotrypsin but were negative for α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. Strain SJ-1 utilizes 3-methyl glucose, methyl pyruvate, L-lactic acid, glucuronamide, acetoacetic acid, and inosine and weakly utilizes other carbon sources (see Table S2 in the supplemental material). Strain SJ-1 resisted antibiotics such as clindamycin, trimethoprim, bacitracin, and β-lactam groups (Table S1). The isolate reduced only nitrate to nitrite but neither one further to ammonia or to gas (N_2, NO, and N_2O) generation. It accumulated polyphosphate and polyhydroxyalkanoate (PHA) granules, which might be relevant to its functionality in WWTPs. Since strain SJ-1 showed a unique phylogenetic position and is very different from other known bacterial species, we concluded that it represents a novel bacterial species and nominated it as *Casimicrobium huifangae* (see Taxonomy below).

Genome, physiology, and metabolism of *C. huifangae*. The complete genome of strain SJ-1 comprises a single circular chromosome of 4,465,898 bp with a GC content of 62.39% (Fig. 1c). No plasmids were detected. There are 4,283 genomic objects, including 4,217 coding gene sequences (CDSs), 46 tRNA replicons, 6 rRNA replicons, 13 misc_RNA replicons, and 1 transfer-messenger RNA (tmRNA) replicon. Two identical copies of 16S rRNA genes of 1,547 bp each are located at bp 4263507 to 4265028 and bp 2831366 to 2832887. About two-thirds (76.22%) of the total CDSs can be classified in at least one COG group: 833 CDSs for cellular processes and signaling, 632 CDSs for information storage and processing, 1,553 CDSs for metabolism, and 913 poorly characterized CDSs (Fig. 1d). There is an incomplete prophage sequence predicted in the genome of strain SJ-1 whose length is 8,918 bp, spanning from bp 3955289 to bp 3964206, with a 63.32% GC content.

To explore the physiology of strain SJ-1, we rebuilt the metabolic pathways and cellular transport systems based on genome annotation (Fig. 2; see Data Set S3 in the supplemental material). Genomic data confirmed that strain SJ-1 is able to grow on mono- and disaccharides, as well as on short-chain fatty acids. Strain SJ-1 did not have genes encoding hydrolytic enzymes active on complex carbohydrates, such as starch, cellulose, and glycogen. A large number of genes encoding sugar permeases and ABC transporters are predicted. Particularly, many genes encoding C_4-dicarboxylate ABC transporter are annotated. It is noteworthy that strain SJ-1 does not carry the genes encoding glucokinase and phosphofructokinase, suggesting an incomplete glycolysis pathway. We noticed that the route that leads the conversion of glyceraldehyde-3-phosphate into erythrose and xylulose and further to fructose-6-phosphate and glucose-6-phosphate is complete. So, it was deduced that the incomplete glycolysis pathway more likely serves for gluconeogenesis, which provides strain SJ-1 with additional substrates for biosynthesis (18). This physiology of strain SJ-1 might indicate that strain SJ-1 is well adapted to its niche in activated sludge of WWTPs, where carbohydrates are depleted but might be rich in fatty acids from bacterial hydrolysis of complex organics as well as from supplementation during operation (such as the addition of acetate for improving nitrogen removal) (19, 20). Proteinaceous substances account for a large proportion of organic load in sewage, and they are easily degraded into short peptides, amino acids, and further to short-chain fatty acids by hydrolytic and
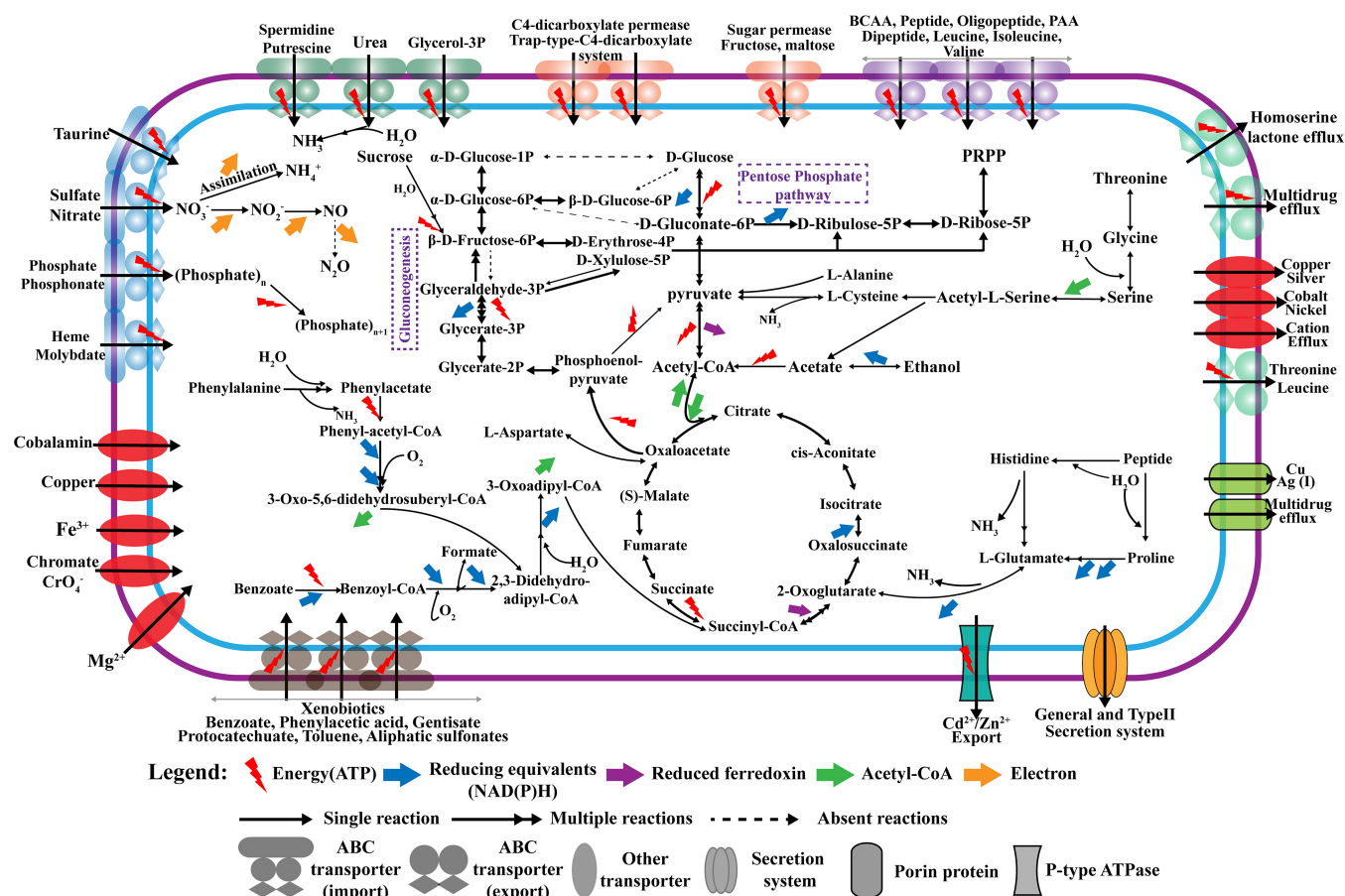
FIG 2 Predicted physiology and metabolic pathways of *C. huifangae* strain SJ-1 based on genome sequences (see Data Set S3 in the supplemental material). BCAA, branched-chain amino acids; PAA, polar amino acids; P, phosphate; PRPP, 5-phospho-alpha-D-ribose-1-diphosphate; NAD(P)H, NAD (phosphate) hydrogen; CoA, coenzyme A.

fermentative bacteria. The genome of strain SJ-1 contains 50 genes encoding ABC transporters and that are potentially involved in the transportation of peptides and amino acids. Those ABC transporters possibly take branched-chain amino acids, polar amino acids, peptides, oligopeptides, leucine, isoleucine, and valine as the substrates. We detected genes encoding enzymes involved in the degradation of peptides and amino acids, such as histidine, phenylalanine, proline, alanine, aspartate, glutamate, serine, cysteine, glycine, and threonine. However, catabolic pathways for amino acids such as lysine, tyrosine, tryptophan, and arginine are incomplete.

Strain SJ-1 might work in the removal of spermidine, putrescine, glutathione, urea, and aromatic compounds in WWTPs. Strain SJ-1 has genes encoding ABC transporters for spermidine, putrescine, glutathione, and urea. These substances are abundant in municipal wastewater, and they might be produced from decomposition of proteins or have resulted from the metabolism of proteins in other organisms (21). Genomic data also predicted that strain SJ-1 might be able to take up and degrade benzoate, phenylacetic acid, gentisate, protocatechuate, toluene, and aliphatic sulfonates. In the genome of strain SJ-1, we found genes encoding benzoate-coenzyme A (benzoate-CoA) ligase (*bclA*), benzoyl-CoA oxygenase, and benzoyl-CoA-dihydrodiol lyase (*boxABCD*) for aromatic ring cleavage. Strain SJ-1 also possesses genes for the degradation of phenylacetate (*paaABKI*) and protocatechuate (*ligAB*). It actually has complete pathways for the degradation of benzoate and phenylacetate (Fig. 2).

Nitrogen and phosphorus removal is critical for WWTP operators (22, 23), and strain SJ-1 might contribute. Genes encoding nitrate transport (*nrtA* and *nasD*), nitrogen regulation sensor (*ntrB*), nitrate reductase (*narGHV*), nitrite reductase (*nirBDS*), and other

proteins (*narJKL*) involved in nitrogen metabolism were annotated. We observed nitrate reduction to nitrite but not further production of gases (N$_2$, NO, or N$_2$O). As for phosphorus removal, strain SJ-1 harbored a number of phosphate transporter and conversion genes (*pstABCS* and *phnEC*), two polyphosphate kinase genes (*ppk*) (24), one exopolyphosphatase gene (*ppx*), and one poly(3-hydroxyalkanoate) polymerase gene (*phaC*); thus, strain SJ-1 is very probably a novel phosphorus-accumulating bacterium (PAO), and this has been verified in the phenotypic experiment. Previous studies disclosed that "*Candidatus* Accumulibacter phosphatis" and *Tetrasphaera* were primary PAOs (25, 26); however, no cultured isolates of "*Candidatus* Accumulibacter phosphatis" were obtained (27). Strain SJ-1 showed 92.37% similarity in terms of the 16S rRNA gene to the yet-to-be cultivated "*Candidatus* Accumulibacter phosphatis."

It seems that strain SJ-1 is well equipped to survive in challenging environments with heavy metals and antibiotic substances, as often detected in municipal WWTPs (28–30). The genes encoding p-type ATPase for Cd^{2+}/Zn^{2+} export, the heavy metal efflux system (Ni, Co, Cu, Ag), and the multidrug efflux system (*mrcA*, *acrAB*, and *oprM*) were annotated in strain SJ-1 genome. Strain SJ-1 carries genes for antibiotic resistance to bacitracin (*uppP*), streptogramin (*vat*), tetracycline (*typA* and *lepA*), kasugamycin (*rsmA*), polymyxin (*yfbG*), aminoglycosides (*aacA*), and macrolides (*macB*) and β-lactams (Data Set S4). In particular, three kinds of β-lactamase-encoding genes were identified in the genome of strain SJ-1, including those encoding β-lactamase class A (*penP*), β-lactamase class C (*ampC*), and β-lactamase OXA-3 (*oxa*).

Strain SJ-1 represents a group of previously uncultured bacteria of the *Betaproteobacteria* and is widely distributed in global WWTPs. Strain SJ-1 was not phylogenetically close (full-length 16S rRNA gene similarity, <92%) to any currently cultivated and validly described bacteria (http://www.bacterio.net). The top hits are members of the *Betaproteobacteria* lineage (Fig. 3), namely *Propionivibrio dicarboxylicus* CreMal1^T (91.68% 16S rRNA gene similarity) of *Rhodocyclales*, *Derxia lacustris* HL-12^T (91.59%) of *Burkholderiales*, *Rhodocyclus tenuis* DSM 109^T (91.53%) of *Rhodocyclales*, *Ideonella sakaiensis* 201-F6^T (91.37%) of *Burkholderiales*, *Leeia oryzae* HW7^T (91.34%) of *Neisseriales*, and *Thiobacillus thiophilus* D24TN^T (90.63%) of *Hydrogenophilales*. Based on the descriptions of *Propionivibrio*, *Derxi*, *Rhodocyclus*, *Ideonella*, *Leeia*, and *Thiobacillus* (31), strain SJ-1 is different from these validly described bacteria in terms of physiological properties, living environments, and phylogenetic position. Thus, we propose that strain SJ-1 represents a novel taxon within the *Betaproteobacteria*.

Although there were no cultivated bacteria closely related to SJ-1, full-length 16S rRNA genes showing high similarity to the 16S rRNA gene of SJ-1 were frequently identified in environmental samples. We downloaded from the NCBI nucleotide collection 51 16S rRNA genes sharing more than 95% sequence identity with strain SJ-1. These 51 sequences were retrieved from diverse environments, and many of them were extracted from activated sludge. The 16S rRNA sequences formed, together with that of strain SJ-1, a deeply branched and coherent cluster within the *Betaproteobacteria* (Fig. 3a). These data indicated that the yet-to-be cultivated members of this novel clade were prevalent in activated sludge and might play a pivotal role in wastewater treatment. We thus propose a new species, *Casimicrobium huifangae*, of a new genus, *Casimicrobium*, within a new family, *Casimicrobiaceae*, to accommodate strain SJ-1 and other yet-to-be cultivated members. A more detailed description is provided in Taxonomy below, and the phylogenetic information for *C. huifangae* is available in Fig. S1.

Recently, a comprehensive investigation of the microbiome of activated sludge was conducted by the GWMC. This study included activated sludge samples from 269 WWTPs in 86 cities, 23 countries, and 6 continents (3). Despite the very large bacterial diversity, there are functionally important global core bacteria in activated sludge, which consists of only 28 core OTUs (taxa) (3). These core taxa are considered to be the "most-wanted" bacteria, and future experimental efforts will be dedicated to understanding and regulating activated sludge processes. After analysis of the representative sequences of the "most-wanted" bacterial core OTUs listed by the GWMC, we found that the representative sequence (253 bp) of core OTU_16 was completely aligned to
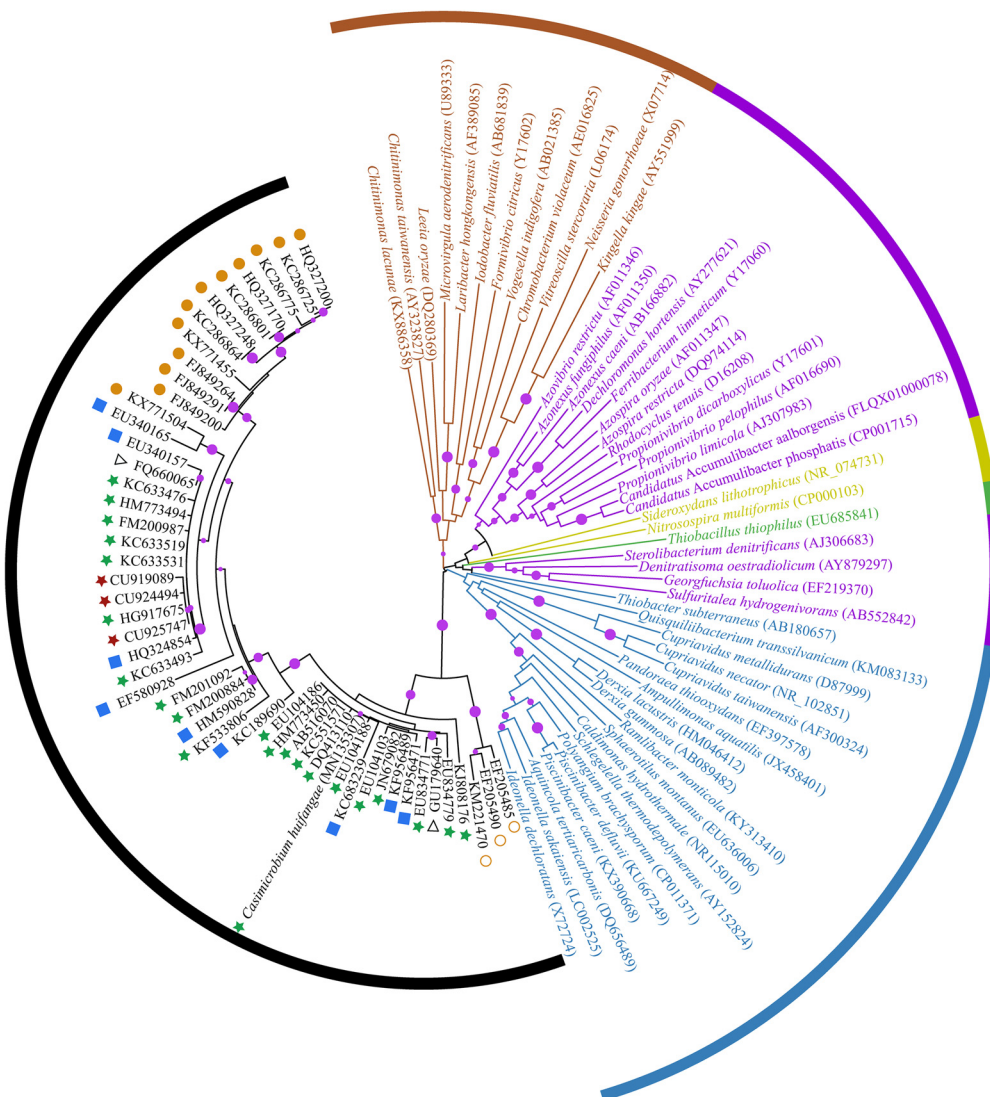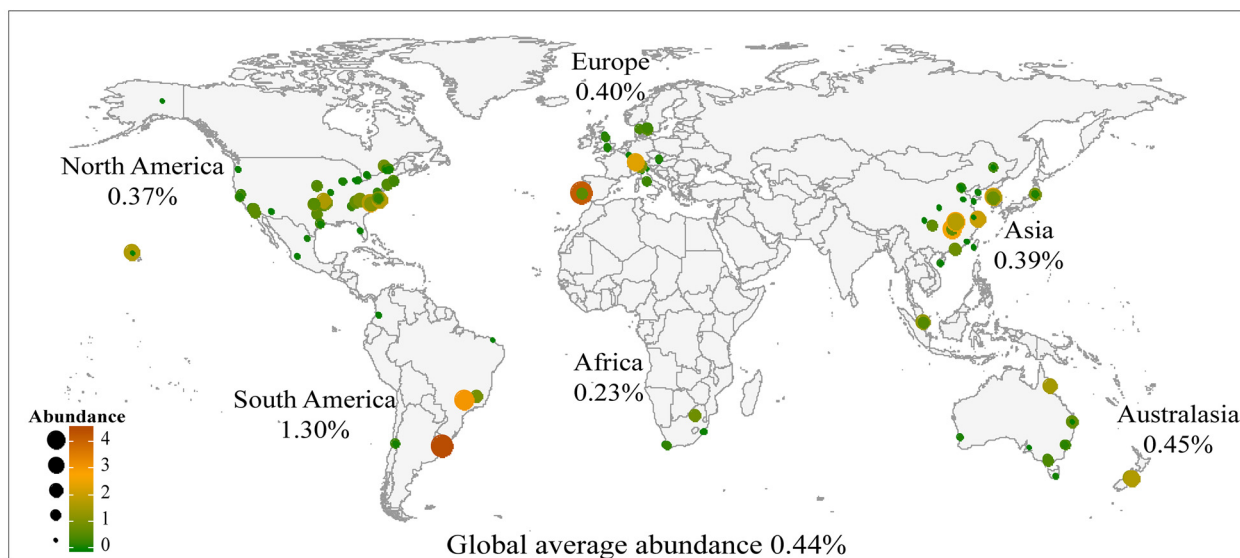
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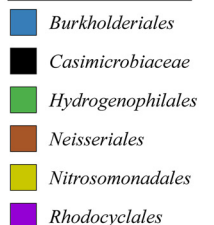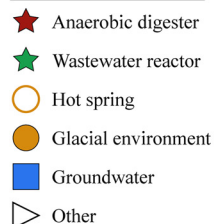
b

FIG 3 The *Casimicrobiaceae* cluster (a) and distribution of *C. huifangae* in global WWTPs (b). (a) Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences, showing the phylogenetic position of *C. huifangae* strain SJ-1 in the *Betaproteobacteria*. GenBank accession numbers are given in

(Continued on next page)

the V4 region of *C. huifangae* strain SJ-1 with 100% similarity. Thus, we concluded that we had successfully cultivated that core OTU_16. The distribution and abundance map of *C. huifangae* is shown in Fig. 3b. The map indicates that the distribution of *C. huifangae* is not restricted from a geographical point of view. The abundance of *C. huifangae* was high in WWTPs located on all continents. The average abundance of *C. huifangae* seemed to be highest in South America (1.30%), followed by Australasia (0.45%), Europe (0.40%), Asia (0.39%), North America (0.37%), and Africa (0.23%). Moreover, *C. huifangae* showed the strongest and most significant positive correlations ($P < 0.001$) with the removal rate of biological oxygen demand (BOD), chemical oxygen demand (COD), total nitrogen (TN), ammonia nitrogen (NH_4^+-N), and total phosphorus (TP) (3). These results supported the importance and functional versatility of *C. huifangae* in the removal of carbon, nitrogen, and phosphate in WWTPs.

Ecological factors that affect the distribution and abundance of *C. huifangae* in WWTPs. In order to identify the ecological factors that affect the distribution and abundance of *C. huifangae* in WWTPs, we collected high-throughput data of the V4 region of 16S rRNA genes from 90 activated sludge samples from Beijing (Qinghe WWTP), Qingdao, Wuxi, Shanghai, and Hong Kong and from 13 WWTPs in Denmark. There were 12,717 OTUs in total, including OTU_11798, which showed 100% identity to the V4 tag of the 16S rRNA gene of strain SJ-1 , and we considered that OTU_11798 represents a *C. huifangae* strain. The distribution of *C. huifangae* in samples from Qinghe WWTPs was investigated. Results showed that *C. huifangae* had abundances of 1.97%, 1.89%, 2.04%, 0.74%, and 1.86% in anoxic and aerobic tanks, influent, foam, and effluent of the anoxic/oxic (A/O) biological process, respectively (Fig. 4a). We noted that the abundance of *C. huifangae* was significantly lower (*t* test, $P < 0.05$) in foaming sludge than in normal sludge. The population of *C. huifangae* showed seasonal variations, with the highest abundance occurring in spring (Fig. 4b).

Data analysis showed that the distribution and population of *C. huifangae* are possibly more affected by environmental factors than by random neutral community assembly mechanisms (32). One environmental factor might be the salt concentration in WWTPs. The organism did not grow in medium with NaCl concentrations higher than 0.5%, and we hardly detected *C. huifangae* in WWTPs from Hong Kong and Qingdao, as it was clearly pointed out that the influent of Hong Kong WWTP contained approximately 1% salinity due to flushing with seawater (6). A second factor might be temperature. The abundance of strain SJ-1 was much higher in spring and summer than in autumn and winter in all sludge samples among the studied WWTPs (Fig. 4b). We observed the highest abundance of strain SJ-1 at about 16°C based on Qinghe WWTP sludge samples (Fig. 5a). A relatively high abundance of SJ-1-like bacteria was observed in other WWTPs at temperatures ranging from 10 to 20°C (Fig. 5b). We observed that strain SJ-1 grew optimally at 30 to 37°C, and the reason for its abundance at a rather low temperature was not understood. One possible reason may be that this bacterium is more competitive at this temperature range (10 to 20°C) than other microbes in the complex microbiome of activated sludge due to its versatile metabolic activities. Another possible reason might be process operation. The operators of Qinghe WWTP used regular supplementation with acetate to improve denitrification efficiency, particularly during spring. Although the growth of strain SJ-1 is faster under aerobic conditions (Fig. 5f), *C. huifangae* was not more abundant at higher dissolved oxygen (DO) concentrations based on data from Qinghe and other WWTPs (Fig. 5d and e). Statistics of worldwide WWTP data showed that *C. huifangae* was more abundant at DO concentrations of about 2.0 mg liter^{-1} (Fig. 5e). Strain SJ-1 in Qinghe WWTP showed high abundance from low (<0.5 mg liter^{-1}) to high (>6 mg liter^{-1}) DO concentrations.

FIG 3 Legend (Continued)
parentheses. Bootstrap percentage values higher than 50% are marked with solid purple circles located in the middle of clades. For currently cultivated and validly described bacteria, different taxa are indicated by different colors (see the key). For 16S rRNA gene sequences of uncultured bacteria, the source environment is indicated by distinct symbols (see the key). The tree scale bar represents 0.01 substitutions per nucleotide position. (b) The GWMC data were used to indicate the distribution of *C. huifangae* (4). Dot sizes and colors represent the abundance of *C. huifangae* as the legend indicates.
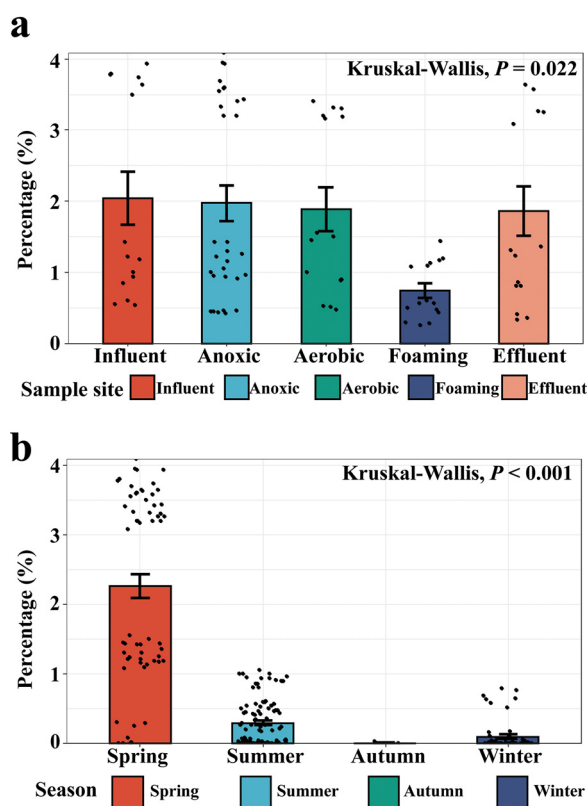
FIG 4 Distribution and seasonal dynamics of *C. huifangae* in WWTPs. (a) Distribution and abundance of *C. huifangae* at different sampling sites of the Qinghe WWTP. (b) Seasonal dynamics of the *C. huifangae* population.

This might be due to the recycling of nitrifying sludge and completely mixed activated sludge in the A/O process of Qinghe WWTP. We observed an increased abundance of *C. huifangae* at pH values ranging from 7.5 to 7.8 in Qinghe WWTP (Fig. 5g) and from pH 7.0 to 7.2 in other WWTPs (Fig. 5h), which is consistent with the optimum pH value (pH range from 7.0 to 7.5) for the growth of strain SJ-1 in pure culture (Fig. 5i).

C. huifangae is an important module hub in the Qinghe WWTP activated sludge bacterial network. To get a better understanding of the ecological roles of *C. huifangae* and how it might interact with other microbes, we further explored the network analysis of strain SJ-1 in the activated sludge microbiome. For 75 normal activated sludge samples of Qinghe WWTP, there were 308 total nodes and 1,211 total links in the network (Table S3). The overall topology indices revealed that the Qinghe WWTP bacterial network connectivity distribution fitted well with the power-law model ($R^2 = 0.741$), indicative of a scale-free network. The average path length (6.683) was higher than the logarithm (2.489) of the total number of network nodes, which proved that these core OTUs in the network were robust and resistant to environmental disturbances. Besides, the Qinghe WWTP microbial network of activated sludge was modular and could be divided into 14 modules with a modularity of 0.730, which was significantly higher than that of its corresponding randomized network (0.306). The Qinghe WWTP microbial network exhibited major network properties of complex systems. As shown in Fig. 6a, four modules were linked together and formed a major network backbone with most of the positive relations. *C. huifangae* was located in module 4 and had extensive links with the other 25 nodes. We divided the total number of nodes into 4 categories (i.e., peripherals, module hubs, connectors, and network hubs) according to within-module connectivity (Z_i threshold value = 2.0) and among-module connectivity (P_i threshold value = 0.4) (Fig. 6b). From ecological perspectives, peripherals might represent specialists, whereas module hubs and connectors are close to gener-
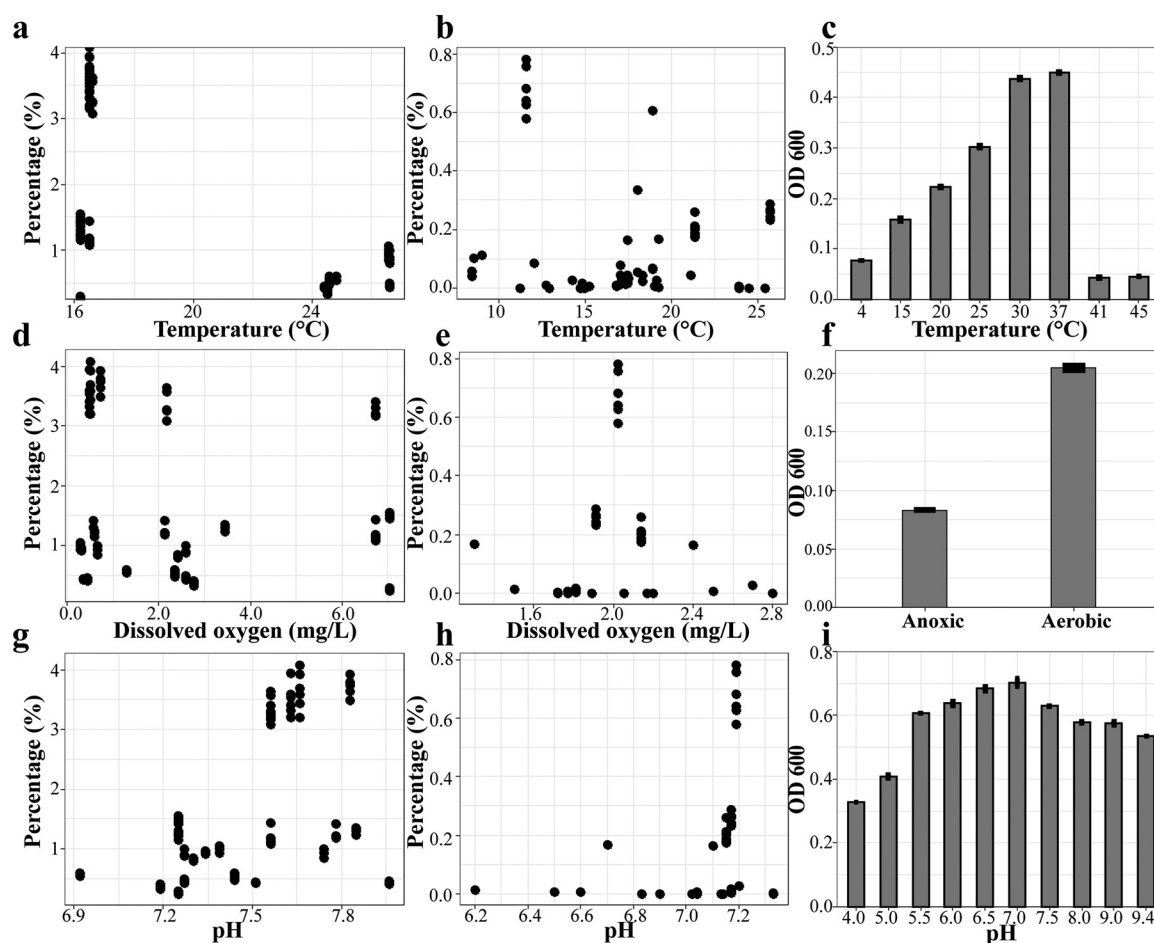
FIG 5 Impact on growth and abundance of *C. huifangae* by environmental factors. (a) The abundance dynamics of *C. huifangae* varied with temperature based on the Qinghe WWTP samples. (b) The abundance dynamics of *C. huifangae* varied with temperature based on other WWTP samples. (c) Growth of pure cultured *C. huifangae* at different temperatures after 12 h of incubation in R2A broth. (d) The abundance dynamics of *C. huifangae* varied with dissolved oxygen concentration based on the Qinghe WWTP samples. (e) The abundance dynamics of *C. huifangae* varied with dissolved oxygen concentration based on other WWTP samples. (f) Growth of pure cultured *C. huifangae* in anoxic and aerobic environments after 12 h of incubation in R2A broth. (g) The abundance dynamics of *C. huifangae* varied with pH values based on the Qinghe WWTP samples. (h) The abundance dynamics of *C. huifangae* varied with pH values based on other WWTP samples. (i) Growth of pure cultured *C. huifangae* at different pH values of R2A broth after 24 h.

alists and network hubs are close to supergeneralists (33). Here, in the Qinghe sludge bacterial network, the majority of nodes (92.9%) were peripherals, with specific functions working within the modules. A total of 12 nodes (3.9%) were module hubs ($Z_i > 2.0$), and 10 nodes (3.2%) were connectors ($P_i > 0.4$). For 12 module hubs, 5 nodes were from *Proteobacteria*, including *C. huifangae* ($Z_i = 2.21$, $P_i = 0$), while other nodes were from *Acidobacteria*, *Chloroflexi*, *Planctomycetes*, *Actinobacteria*, and *Bacteroidetes*. For 10 connectors, 6 nodes were from *Proteobacteria*, while other nodes from *Planctomycetes*, *Nitrospirae*, and *Patescibacteria*. These results demonstrated the important roles of *Proteobacteria* in the microbiome of municipal activated sludge, as well as some low-abundance phyla that may be indispensable in the activated sludge bacterial network. The 25 neighbors of *C. huifangae* included members of *Acidobacteria*, *Armatimonadetes*, *Actinobacteria*, *Bacteroidetes*, *Planctomycetes*, *Proteobacteria*, and *Verrucomicrobia* (Data Set S5). Among them, most neighbors (17) were *Proteobacteria*, especially *Betaproteobacteria*, including *Zoogloea* species, a necessary and significant zoogloeal floc former in activated sludge (34, 35). The three neighboring *Bacteroidetes* nodes were all classified in the *Chitinophagaceae* family. In addition, there were three negative interactions between *C. huifangae*, with the only *Actinobacteria* node (*Microtrichaceae*) and with two *Woodsholea* nodes. More networking of *C. huifangae* with
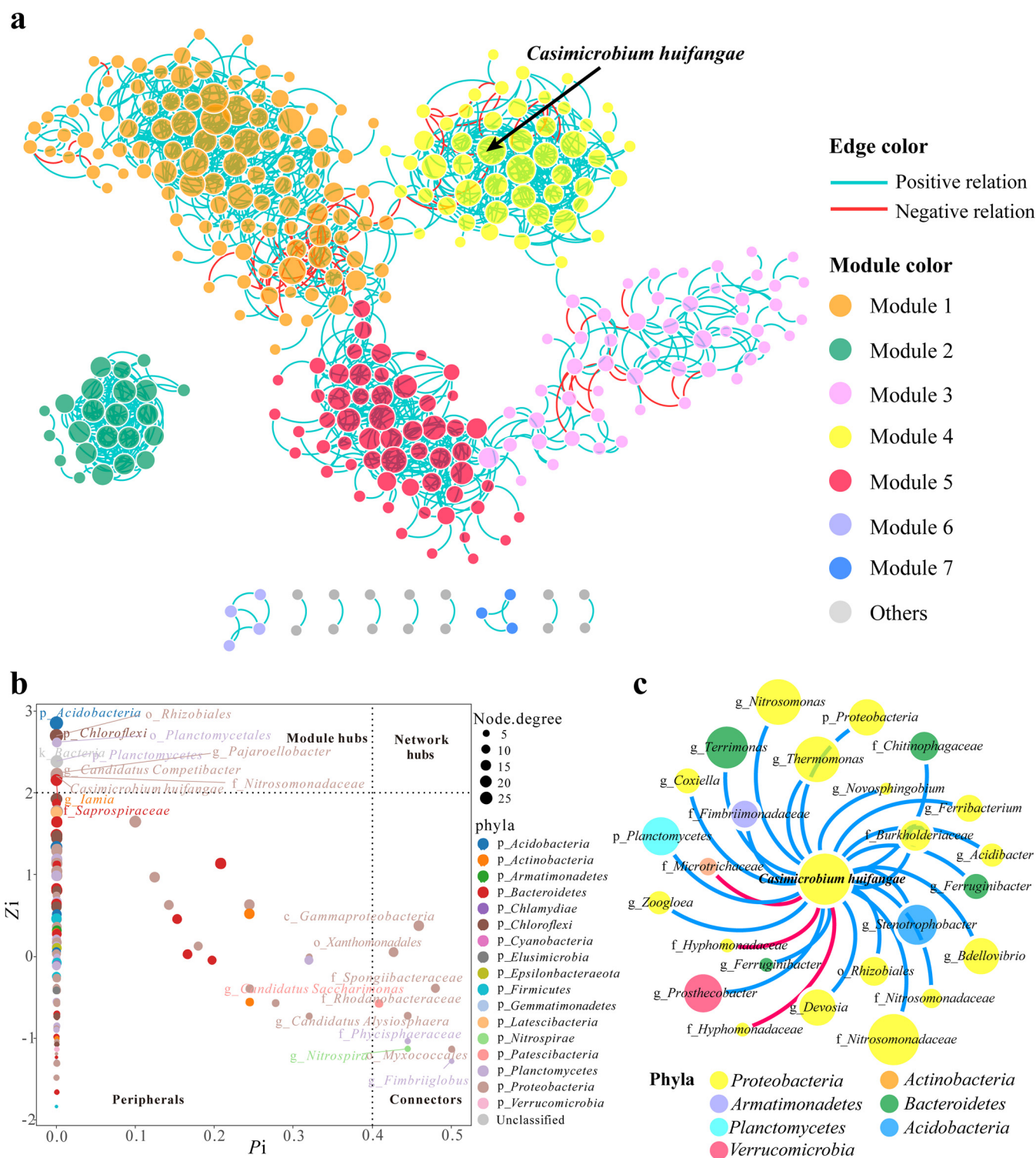
FIG 6 Characteristics of the bacterial network of *C. huifangae* in the Qinghe WWTP built by 75 normal activated sludge samples. (a) Total network graph with module structure determined by the fast greedy modularity optimization method. Each node represents an OTU in the Qinghe WWTP. The colors of nodes indicate different modules, blue edges indicate positive interactions between two individual nodes, and red edges indicate negative interactions. (b) ZP plot showing the distribution of OTUs based on their topological roles in the Qinghe WWTP bacterial network. Each color represents a phylum, and the dot size represents the node degree (connectivity). The topological role of each OTU was determined according to the scatter plot of within-module connectivity (Z_i) and among-module connectivity (P_i). (c) Cooccurrence network between *C. huifangae* (OTU_11798) and its adjacent, first-level OTUs in the Qinghe WWTP bacterial network. The nodes representing OTUs were annotated by their lowest classification level, and node sizes are displayed according to their degree in the network. Different colors of nodes indicate their phyla.

other bacterial nodes is visualized in Fig. 6c, suggesting its central role in the Qinghe WWTP bacterial network.

Conclusions. The "most-wanted" core bacterium *C. huifangae* in activated sludge has been successfully cultivated, genome sequenced, and phenotypically characterized. This globally distributed bacterium, *C. huifangae*, serves as a central hub in the network of the Qinghe WWTP activated sludge microbiome. *C. huifangae* is predicted to be functionally important for nitrogen and phosphorus removal from wastewater. Phylogenetic, genomic, and phenotypic characterization of strain SJ-1 revealed its standing as a novel species (namely *Casimicrobium huifangae*) of a new genus (*Casimicrobium*) and a new family (*Casimicrobiaceae*) within the *Betaproteobacteria* class. *C. huifangae* occurs widely in activated sludge, and its distribution is affected by ecological factors such as salt and DO concentrations, temperature, and pH of WWTPs.

TAXONOMY

Description of *Casimicrobiaceae* fam. nov. *Casimicrobiaceae* (Ca.si.mi.cro'ba.ce.ae. N.L. neut. n. *Casimicrobium*, type genus of the family; suff. -*aceae*, ending to denote a family; N.L. neut. pl. n. *Casimicrobiaceae*, the family of the genus *Casimicrobium*). According to 16S rRNA gene sequence analysis, the family belongs to the class *Betaproteobacteria*. Currently, the family has the same description as that for the following genus. The type genus is *Casimicrobium*.

Description of *Casimicrobium* gen. nov. *Casimicrobium* (Ca.si.mi.cro'bi.um. N.L. neut. n. *microbium* a microbe; N.L. neut. n. *Casimicrobium*, a microbe named in honor of CAS, the Chinese Academy of Sciences). Cells are aerobic, Gram negative, rod shaped, nonmotile with no flagella, do not sporulate, and are oxidase positive and catalase positive. The major fatty acids are $16:1\omega7c/\omega6c$, 16:0 and 14:0 iso. The type species is *Casimicrobium huifangae*.

Description of *Casimicrobium huifangae* sp. nov. (hui.fan'gae. N.L. gen. n. *huifangae* named in honor of Hui-Fang Yang, a Chinese microbiologist who did pioneering work on environmental microbiology). Displays the following properties in addition to those given in the previous genus description. Colonies are small, approximately 0.5 to 0.8 mm in width, cream in color, semitransparent with regular circular margins and wet convex appearance after 3 days of incubation at 30°C. Most cells are 0.7 to 0.8 μm in width and 1.5 μm in length. Good growth occurs in R2A medium between 30 and 37°C; no growth occurs at temperatures higher than 41°C. Strain SJ-1^T can stand pH from 5.5 to 9.4 (optimum, pH 7.0 to 7.5) and NaCl concentration (wt/vol) from 0% to 1% (optimum, 0%). According to the API ZYM and API 20NE systems and genome analysis, gelatinase, alkaline phosphatase, acid phosphatase, esterase (C_4), esterase lipase (C_8), leucine arylamidase, valine arylamidase, cystine arylamidase, naphthol-AS-BI-phosphohydrolase, trypsin, and α-chymotrypsin were found positive, while lipase (C_{14}), tryptophanase, arginine dihydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-fucosidase, and α-mannosidase were found negative. Although it may encode enzymes for hydrolyzing urea, urease activities were found negative. Casein and Tween 20 can be hydrolyzed, while cellulose, starch, tyrosine, and Tween 40, Tween 60, and Tween 80 cannot be hydrolyzed. Strain SJ-1 was found to strongly utilize 3-methyl glucose, methyl pyruvate, L-lactic acid, glucuronamide, acetoacetic acid, and inosine (see Table S2 in the supplemental material). Strain SJ-1^T reduced only nitrate to nitrite and neither of these further to ammonia or to gas (N_2, NO and N_2O) generation. Polyphosphate and polyhydroxyalkanoate (PHA) can be stored as granules in the cells. Cells are sensitive to (given in micrograms per disc, unless otherwise indicated) streptomycin (10), polymyxin (300 IU), ciprofloxacin (5), neomycin (30), rifampin (5), erythromycin (15), vancomycin (30), gentamicin (10), chloramphenicol (30), azithromycin (15), spectinomycin (100), tetracycline (30), kanamycin (30), tobramycin (10), netilmicin (30), penicillin (10 IU), ampicillin (10), piperacillin (100), carbenicillin (100), cefalexin (30), cefadroxil (30), cefazolin (30), cefalotin (30), cefprozil (30), and cefoxitin (30). The genome GC content of the type strain SJ-1 is 62.39%. The type strain, SJ-1^T (=CGMCC

1.30039^T = KCTC 72612^T), was isolated from activated sludge of the Qinghe WWTP, Beijing, China.

MATERIALS AND METHODS

Activated sludge sampling, isolation, and cultivation of bacteria. In total, 90 activated sludge samples were collected from the anoxic/oxic (A/O) process tank of the Qinghe WWTP located in Beijing, China (116°21′47.25′′E, 40°02′35.54′′N), in March 2017, June 2017, August 2017, and March 2018. Sludge samples covered influent, anoxic, aerobic, foaming, and effluent parts of the A/O tank. The performances of the Qinghe WWTP at sampling times are shown in Data Set S1 in the supplemental material. All samples were collected in triplicate in sterilized containers and kept in a portable icebox before being quickly transported to the Environmental Microbiology Research Center (EMRC) at the Institute of Microbiology, CAS. Each sample was divided into two parts, one of which was stored at 4°C for bacterial isolation and cultivation and the other of which was stored at −80°C prior to DNA extraction.

The sludge samples used for bacterial isolation and cultivation were pretreated by means of gentle sonication and serial dilution by a phosphate-buffered saline (PBS) buffer solution. Aliquots of dilutions of 10^{-5}, 10^{-6}, and 10^{-7} were plated onto six different culture media: (i) 10-fold diluted R2A agar (36); (ii) sewage agar (1,000 ml sterile sewage and 15 g agar, autoclaved at 121°C for 15 min); (iii) ammonia oxidation agar [$(NH_4)_2SO_4$, 2.0 g; K_2HPO_4, 0.75 g; NaH_2PO_4, 0.25 g; $MgSO_4·7H_2O$, 0.03 g; $NaCO_3$, 5.0 g; $MnSO_4·4H_2O$, 0.01 g; 15 g agar, and 1,000 ml distilled water, with pH adjusted to 7.2]; (iv) nitrite oxidation agar ($NaNO_2$ 1.0 g; K_2HPO_4 0.75 g; NaH_2PO_4, 0.25 g; $MgSO_4·7H_2O$, 0.03 g; Na_2CO_3, 5.0 g; $MnSO_4·4H_2O$, 0.01 g; 15 g agar, and 1,000 ml distilled water, with pH adjusted to 7.2); (v) denitrification agar (sodium citrate, 5.0 g; KNO_3, 2.0 g; KH_2PO_4, 1.0 g; $MgSO_4·7H_2O$, 0.2 g; Na_2CO_3, 5.0 g; $K_2HPO_4·3H_2O$, 0.01 g; 15 g agar, and 1,000 ml distilled water, with pH adjusted to 7.2); (vi) PAM agar (37). The plates were incubated at both 15°C and 30°C under the aerobic conditions. Individual colonies appearing on plates over a period of 4 weeks were continuously picked up and transferred to new corresponding agar plates. All isolates were further purified by repeated streaking until pure cultures were obtained. The purity of the isolates was checked by 16S rRNA gene sequencing. The nearly full-length 16S rRNA gene was amplified using the 27F and 1492R primers (38).

Genomic DNA extraction and genome sequencing. The total genomic DNA of strain SJ-1 was extracted using the Wizard Genomic DNA purification kit by following the manufacturer's instructions. DNA quantity and quality were determined with a NanoVue Plus spectrophotometer (GE Healthcare, USA). Genome sequencing was performed using both the Illumina HiSeq 4000 platform and the PacBio RS II platform at BGI (Shenzhen, China). For Illumina libraries, the insert size was 270 bp with a pair-end sequencing length of 150 bp. After filtering and cutting the adapters, there were 1,136 Mb (254×) clean data from Illumina sequencing. For the PacBio platform, subreads (length < 1 kb) were removed with the program Pbdagcon (https://github.com/PacificBiosciences/pbdagcon) for self-correction. There were 1,169 Mb (261×) of reliable corrected reads from the PacBio data set assembled by Celera Assembler (https://sourceforge.net/projects/wgs-assembler/files/wgs-assembler/wgs-8.3/). After that, the GATK was used for single-base corrections in the Illumina clean data (https://software.broadinstitute.org/gatk/) to improve the accuracy of assembling. Finally, we obtained the complete circular genome of strain SJ-1 with a length of 4,465,898 bp.

Community profiling with 16S rRNA gene amplicon sequencing. One milliliter of each activated sludge sample was centrifuged at 16,000 × g for 10 min at 4°C. Total DNA was extracted from 0.25 g (wet weight) of pellet by using a PowerSoil DNA isolation kit (Qiagen, Inc., Germany) in accordance with the manufacturer's protocol. Purified DNA was used as the PCR templates for 16S rRNA gene amplification of the hypervariable V4 region with primer pair 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) containing barcodes at the 5′ end. The obtained purified PCR products were quantified, pooled for library construction, and sequenced at BGI with the Illumina HiSeq 2500 platform. All of the paired-end 250-bp fastq sequences were deposited in the NCBI SRA database under BioProject accession number PRJNA550218. We also downloaded high-throughput sequencing data of the V4 region of the 16S rRNA gene from other research papers investigating bacterial communities in WWTPs (6, 39–41). We cut primers for all of these raw sequences by using Cutadapt software (42), and then the sequences were further filtered to obtain accurate and reliable clean data by fastp software (43) with the following criteria: (i) truncate sequence reads of low quality (Q score < 20) with a 25-bp sliding window and trim final reads to less than 75% of their original length; (ii) remove reads contaminated by adapters; (iii) remove reads with ambiguous bases (N base). Then FLASH software (44) was used to merge overlapped paired-end reads into tags. The OTUs at the 97% similarity threshold of all tags were generated by USEARCH (45), and singletons, chimeras, and nonprokaryote sequences were removed using the reference database Silva 132 SSU (46). The representative sequences of OTUs were annotated using RDP Classifier (47) with a confidence cutoff of 80% by using three databases, including the RDP 16S rRNA gene database (release 11.5) (48), the SILVA SSU 132 database (46), and MiDAS (Microbial Database for Activated Sludge) (49). The OTU table is shown in Data Set S2.

To identify the OTU that represents *C. huifangae*, a local BLAST search (50) was conducted with more than 97% similarity and at least 200-bp alignment across the query V4 sequences. In addition, in order to understand the ecological distribution of *C. huifangae* on a global scale, we downloaded the representative sequences of the most extensive investigation of an activated sludge bacterial community from the GWMC website (http://gwmc.ou.edu/data-disclose.html) (3). We constructed phylogenetic molecular ecological networks (pMENs) between *C. huifangae* and other OTUs in the Qinghe WWTP activated sludge by use of the Molecular Ecological Network Analysis (MENA) pipeline (33) with a cutoff threshold of 0.93 according to the random matrix theory. The 781 OTUs consistently appearing in a total

of 75 normal Qinghe sludge samples (excluding foaming samples) were picked up, and a uniform transformation was used to obtain the Pearson correlation matrix. Modules of network nodes were separated according to greedy modularity optimization (51), and Cytoscape (v3.7.1) was used for network visualization (52).

Phenotypic and biochemical tests of *C. huifangae*. To check the growth on different media, *C. huifangae* strain SJ-1 was cultured on R2A agar (Difco), Trypticase soy agar (TSA; Difco), nutrient agar (NA; Difco), and LB agar (Difco). Strain SJ-1 was routinely cultivated on R2A medium for subsequent phenotypic and biochemical tests. Tests for Gram staining, oxidase, catalase, and hydrolysis of casein, carboxymethyl cellulose, starch, tyrosine, and Tween 20, 40, 60, and 80 were done according to the methods of Smibert et al. (53). Cell morphology was examined by scanning electron microscopy (QUANTA 200; FEI) and transmission electron microscopy (JEM-1400; JEOL) using cells cultivated for 5 days on R2A medium. Motility was tested by incubating SJ-1 cells in soft R2A agar that contained 0.5% (wt/vol) agar at 25°C. The effects of temperature (4, 15, 20, 25, 30, 37, 41, and 45°C), NaCl concentration (0, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0% [wt/vol]), and pH (from 4 to 10 at intervals of 1 pH unit) on growth were evaluated after 7 days of incubation in R2A broth. Solution pH was adjusted by acetate buffer (0.1 M; for pH 4.0 to 6.0), phosphate buffer (0.1 M; for pH 6.0 to 8.0), and carbonate buffer (0.1 M; for pH 8.0 to 10.0). Cell growth was estimated by measuring turbidity at 600 nm using a UV/visible spectrophotometer (Specord 205; Analytik Jena). Strict anaerobic growth was tested in an anaerobic tube with R2A broth supplemented with L-cysteine (1 g liter^{-1}) and resazurin (1 mg liter^{-1}) as the reductant and oxygen indicators, respectively, and the upper air layer was replaced by nitrogen gas. Anoxic growth was tested using R2A broth under static culture at a temperature of 30°C. Antibiotic resistance tests were conducted using the agar diffusion method with antibiotic-impregnated discs (Beijing SanYao Science & Technology Development Co.). The antibiotics and doses used are listed in Table S1. Other enzyme activities and biochemical properties were determined using the commercial systems API 20NE (bioMérieux), API ZYM (bioMérieux), and Biolog GEN III MicroPlate according to the manufacturers' instructions. For the nitrate reduction test, strain SJ-1 was cultured using R2A broth supplemented with KNO_3 (100 mg N/liter) and sealed with liquid paraffin and Vaseline (1:1 [vol/vol]). Polyphosphate granules were visualized using the Neisser stain (54), and polyhydroxyalkanoate (PHA) granules were observed using the Nile blue A stain (55).

Phylogenetic and genomic analysis. Genomic analysis of *C. huifangae* strain SJ-1 was performed using the MicroScope annotation pipeline (56). After gene prediction with Prodigal (57), annotations were curated automatically for all genes using BLASTP (50), searching against the COG (58) and EggNOG (59) protein orthologous group databases. The annotation criteria were as follows: (i) at least 40% amino acid identity to classify homologs and (ii) at least 25% identity to determine putative homologs (60). The enzymatic classification was based on UniProt (61) and PRIAM (62) results. SignalP was used to predict protein localization (63). Metabolic pathways of *C. huifangae* strain SJ-1 were according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, adjusted by the KAAS annotation server (64, 65). The ARDB database was used to understand its antibiotic resistance (66), and PHAST was used to predict prophage (67). In addition, after the full-length 16S rRNA gene sequence was obtained by RNAmmer under GenBank accession number MN135307 (68), the phylogenetic neighbors and sequence similarities were calculated using the EzBioCloud server (69) and the NCBI Nucleotide collection. Multiple sequence alignments were performed using the MUSCLE program (70). The phylogenetic tree was reconstructed using the MEGA7 program (71) and iTOL (72), with the neighbor-joining method fitted by Kimura's two-parameter model with consideration of transitions and transversions (73, 74). Bootstrap analysis was used to evaluate the confidence level of the branch nodes by performing 1,000 replicates (75).

Data availability. *Casimicrobium huifangae* strain SJ-1 is deposited in the China General Microbiological Culture Collection Center (CGMCC) under accession number CGMCC 1.30039 and the Korean Collection for Type Cultures (KCTC) under accession number KCTC 72612. The genome sequence of SJ-1 is available in the NCBI database under accession number CP041352. The sequencing data of Qinghe WWTP activated sludge are available in the NCBI SRA database under accession number PRJNA550218. Other publicly available data on which the conclusions of the paper rely are available from the NCBI SRA database under accession numbers SRP053365, PRJNA324303, PRJEB5095, and SRP101823. The OTU tables and representative sequences of the OTUs of the global activated sludge bacterial community by the GWMC are available on the GWMC website (http://gwmc.ou.edu/data-disclose.html).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.
SUPPLEMENTAL FILE 2, XLSX file, 0.03 MB.
SUPPLEMENTAL FILE 3, XLSX file, 9.2 MB.
SUPPLEMENTAL FILE 4, XLSX file, 0.1 MB.
SUPPLEMENTAL FILE 5, XLSX file, 0.02 MB.
SUPPLEMENTAL FILE 6, XLSX file, 0.01 MB.

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