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# *Alteromonas flava* sp. nov. and *Alteromonas facilis* sp. nov., two novel copper tolerating bacteria isolated from a sea cucumber culture pond in China

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ABSTRACT

Two bacterial strains, P0211$^T$ and P0213$^T$, were isolated from a sea cucumber culture pond in China. The strains were able to resist high copper levels. These two strains were characterized at the phenotypic, chemotaxonomic, and genomic level. They were completely different colors, but the 16S rRNA genes showed 99.30% similarity. Phylogenetic analysis based on the sequences of the 16S rRNA gene and five housekeeping genes (*dnaK*, *sucC*, *rpoB*, *gyrB*, and *rpoD*) supported the inclusion of these strains within the genus *Alteromonas*, and the two isolated strains formed a group separated from the closest species *Alteromonas aestuariivivens* KCTC 52655$^T$. Genomic analyses, including average nucleotide identity (ANIb and ANIm), DNA–DNA hybridization (DDH), and the percentage of conserved proteins (POCP), clearly separated strains P0211$^T$ and P0213$^T$ from the other species within the genus *Alteromonas* with values below the thresholds for species delineation. The chemotaxonomic features (including fatty acid and polar lipid analysis) of strains P0211$^T$ and P0213$^T$ also confirmed their differentiation from the related taxa.

The results demonstrated that strains P0211$^T$ and P0213$^T$ represented two novel species in the genus *Alteromonas*, for which we propose the names *Alteromonas flava* sp. nov., type strain P0211$^T$ (= KCTC 62078$^T$ = MCCC 1H00242$^T$), and *Alteromonas facilis* sp. nov., type strain P0213$^T$ (= KCTC 62079$^T$ = MCCC 1H00243$^T$).



## Introduction

Bacteria of the genus *Alteromonas* represent one of the oldest known genera of Gram-negative, strictly aerobic, heterotrophic bacteria with one polar flagellum [2]. The taxonomy of this genus underwent a number of revisions and reclassifications, leaving *Alteromonas macleodii* as the single representative species of the genus for about a decade [8]. Currently, the number of validly described species have reached 18 species (http://www.bacterio.net/alteromonas.html). 16S rRNA gene sequence analysis is primary tool for delineation of novel bacterial species. However, with the increasing amount of new species being identified, it is becoming apparent that the threshold value of 97% 16S rRNA gene sequence similarity may not be as accurate as previously thought [21]. As a result, Jun et al. [9] and Konstantinidis et al. [10] proposed that Multilocus Phylogenetic Analysis (MLPA) generated data was useful complementary techniques for the description of new *Alteromonas* species; their phylogenetic analysis, incorporating five house-keeping genes (*dnaK*, *sucC*, *rpoB*, *gyrB*, and *rpoD*), revealed a threshold value of 98.9% that could be considered as the species cut-off value for the delineation of *Alteromonas* spp.

Copper is an essential heavy metal trace element; however, excess of this metal results in cell damage. To cope with copper toxicity, bacteria have evolved various copper resistance genes, including, the copper resistance operon, *copABCD* [14,15], the copper transport genes, *cutABCDEF* [19], the *cus* determinant of cation efflux of copper, *cusCFBA* [6], and the copper-responsive regulatory system, CueR-, CusSR-, CpxAR-, and YedVW [24]. A mutation in one or more of these genes results in increased copper sensitivity [15,19,6,24].

In this study, two isolates obtained from a sea cucumber culture pond were phenotypical, chemotaxonomical, and genetically characterized to determine their taxonomic position. In addition, the

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strains were able to resist high copper levels, and we explored the existence of genes related to copper tolerance by analyzing their genomes.

## Material and methods

### *Organism, growth and maintenance conditions*

A sediment sample was collected from a sea cucumber culture pond located in Rongcheng, Shandong province, China (122° 14′ E, 36° 54′ N). The sample was diluted with 9 ml sterile seawater and spread onto marine agar 2216 (MA; Becton Dickinson). Subsequently, a yellow-colored strain $P0211^T$ and a beige-colored strain $P0213^T$ were isolated on MA and subsequently maintained and subcultivated on MA at 37 °C. They were then stored at −80 °C in sterile 1% (w/v) saline supplemented with 15% (v/v) glycerol.

### *Morphological, physiological and biochemical analysis*

Colony morphologies of the strains were observed after 48 h of incubation on MA. The Gram reaction was determined using the bioMérieux Gram-stain kit according to the manufacturer's instructions. Gliding motility was determined as described by Bowman [3]. Type of flagellation and cell morphology was documented using light microscopy (E600; Nikon) and transmission electron microscopy (Jem-1200; Jeol). The temperature range for growth was examined on MA medium at 4, 15, 20, 25, 28, 30, 33, 37, 40, 45, and 50 °C. The pH range for growth was investigated between pH 5.5 and 9.5 (in increments of 0.5 pH units) in marine broth 2216 (MB; Becton Dickinson) at 37 °C containing the following buffers (Sangon): MES (pH 5.0, 5.5, and 6.0), PIPES (pH 6.5 and 7.0), HEPES (pH 7.5 and 8.0), tricine (pH 8.5), and CAPSO (pH 9.0, 9.5, and 10.0), at concentrations of 20 mM; then the $OD_{600}$ was measured. The effect of NaCl concentrations on growth was examined on modified marine ZoBell agar and in modified marine ZoBell broth made with 0.5% peptone, 0.1% yeast extract, 0.01% $FePO_4$, artificial seawater (consisting of 0.32% $MgSO_4$, 0.22% $MgCl_2$, 0.12% $CaCl_2$, 0.07% KCl, 0.02% $NaHCO_3$, w/v) and in the presence of 0.0–10.0% (w/v) NaCl at intervals of 1.0%. A 1 M copper sulfate stock solution was prepared in deionized water, filter-sterilized through a 0.2 μM nitrocellulose filter, and stored in sterile, polycarbonate tubes. The effect of $Cu^{2+}$ concentrations on growth was examined on MB and in the presence of 0.0–1.2 mM copper sulfate at intervals of 0.1 mM. Colony growth was recorded every 12 h. Growth on MA plate under anaerobic (10% $H_2$, 10% $CO_2$, and 80% $N_2$) and microaerobic (5% $O_2$, 10% $CO_2$, and 85% $N_2$) conditions were determined after incubation for 14 days in an anaerobic jar with or without 0.1% (w/v) $KNO_3$. Oxygen indicator (MGC, C-22) was added in the anaerobic jar. For the accuracy of the experiment, we selected a facultative anaerobic bacteria (*Draconibacterium orientale* DSM $25947^T$) as a positive control, and a strictly aerobic bacteria (*A. macleodii* ATCC $27126^T$) as a negative control.

Reduction of nitrate, catalase, oxidase and lipase (Tweens 20, 40, 60, and 80) activities, and hydrolysis of agar, starch, casein, alginate, and cellulose were tested according to Dong and Cai [26]. The substrate-oxidation profile was obtained using Biolog GEN III microplates following the manufacturer's protocol. API 50 CHB fermentation kits (bioMérieux) were used for determining acid production from carbohydrates. Various biochemical tests and additional enzyme activities were determined using API 20E strips and the API ZYM system (bioMérieux). Each set of experiments was repeated at least twice.

### *Phylogenetic analysis*

The 16S rRNA gene was amplified by PCR with two universal primers 27f and 1492r, according to the method described by Liu et al. [12]. The amplified gene was ligated into the pMD18-T vector (Takara) and recombinant plasmids were reproduced in *Escherichia coli* DH5α cells. The nearly complete 16S rRNA gene sequence was compared with those held in databases using BlastN and EzBioCloud's Identify service. MLPA analysis of the partial sequences of five housekeeping genes more commonly used in the phylogenetic analysis of *Alteromonas* species (*dnaK*, *sucC*, *rpoB*, *gyrB*, and *rpoD*) was conducted for detailed identification of the strains. Sequences of strains $P0211^T$ and $P0213^T$ were obtained from their genome, and sequences from other species of the genus *Alteromonas* were obtained from GenBank. Accession numbers of all the sequences used in this work are listed in Table S1. The sequences were concatenated in the following order: *dnaK*–*sucC*–*rpoB*–*gyrB*–*rpoD*. This realignment resulted in a sequence of 3350 bp in length. Phylogenetic analysis of the 16S rRNA gene and five housekeeping genes was conducted with the MEGA software version 7.0 [11], using Neighbor-joining (NJ), Maximum Likelihood (ML) algorithms and Maximum-parsimony (MP) with a bootstrap of 1000 replications [5].

Genomic DNA purification, sequencing, assembly, annotation, and analyses of G + C content of DNA

Genomic DNA of strains was extracted and purified using a bacterial genomic DNA mini kit (TaKaRa Bio). The draft genomes of two isolates were sequenced by Genewiz (Suzhou, China) using Illumina Hiseq sequencing technology. Assembly was done using Velvet [25] and genes were identified using Prodigal [7]. The predicted CDSs and functional annotation were translated and used to search the National Center for Biotechnology Information (NCBI) non-redundant database, as well as KEGG, COG, GO databases, and the RAST Server [1]. The DNA G + C mol% content of the two strains was determined from the mean G + C content of the draft genome.

### *Genome comparison*

In order to provide support for the classification of two strains as novel species within the genus *Alteromonas*, the Average Nucleotide Identity (ANI), using BLAST (ANIb) and MUMmer (ANIm) algorithms, was calculated using the software Jspecies (V1.2.1) [18]. DNA–DNA hybridization (DDH) was calculated *in silico* by the Genome-to-Genome Distance Calculator (GGDC 2.1) [13] using the BLAST method. Results were based on recommended formula 2 (identities/HSP length), which is independent of genome length and is thus robust against the use of incomplete draft genomes. The percentage of conserved proteins (POCP) was calculated according to Qin et al. [17].

### *Chemotaxonomic analysis*

For chemotaxonomic analyses, biomass of the two isolated strains and reference strains (*Alteromonas aestuariivivens* KCTC $52655^T$, *A. macleodii* ATCC $27126^T$, and *Alteromonas lipolytica* MCCC $1K03175^T$) was harvested from cultures after incubation in MB at 37 °C for 16 h, when bacteria were in the late stage of logarithmic growth phase. Fatty acids were extracted according to the standard protocol of MIDI (Sherlock Microbial Identification System). Fatty acids were methylated and analyzed by an Agilent 6890N gas chromatograph and identified using the TSBA40 database of the microbial identification system [20]. Polar lipids were separated by two-dimensional silica gel TLC. Total lipids were detected using molybdatophosphoric acid, and defined functional groups were detected using spray reagents specific for the groups. Full details are given by Tindall et al. [22].

## Results and discussion

Both strains were Gram negative, rod-shaped, and motile by one polar flagellum (Fig. S1). Cells of strains P0211T and P0213T were 0.4–0.8 μm and 0.3–0.6 μm in width, and 0.8–3.6 μm and 1.0–4.2 μm in length, respectively. Growth of the two strains was found to occur between 15–45 °C (optimum 37 °C), pH 6.5–8.5 (optimum pH 8.0–8.5), and in the presence of 1.0–7.0% (w/v) NaCl (optimum 2.0%). Growth of strains P0211T and P0213T occurred in the presence of 0.0–0.6 mM copper sulfate and 0.0–1.0 mM copper sulfate, respectively. Growth of strain P0213T occurred in anaerobic and microaerobic conditions with or without nitrate, but strain P0211T did not; this indicated that strain P0211T was aerobic, while strain P0213T was facultatively anaerobic. The strains tested shared the main properties of the genus *Alteromonas*: (i) Gram-negative, rod-shaped, motile by a polar flagellum, (ii) positive for oxidase, alkaline phosphatase, esterase lipase (C8), and leucine arylamidase activities, (iii) negative for indole production, and acid production from D-mannose, inositol, and D-sorbitol, and activity of *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. The isolates showed high phenotypical homogeneity, although variable reactions were observed for several traits among other species tested (Table 1). The strain P0213T differed from strain P0211T in the ability of nitrate reduction. It is known that there are two distinguishable types of nitrate reductases, the periplasmic nitrate reductases (Nap) and the membrane-bound nitrate reductases (Nar). The genome analysis of the strain P0213T demonstrated the existence of genes of Nar synthesis, *narGHIJ*, and Nar was the sole nitrate reductase (Table S2); however, we did not find any nitrate reductase genes in the genome of the strain P0211T. The phenotypic characterization was consistent with the genome analysis. The strains P0211T and P0213T differed from the closest related species in relation to the presence of trypsin and α-chymotrypsin activities, hydrolysis of starch, and not producing acid from D-xylose and cellobiose (Table 1). Further data on the morphological, physiological, and biochemical characteristics of the isolates are given in the species descriptions and Table 1.

Nearly full length 16S rRNA gene sequences (>1400 bp) were obtained for strain P0211T (MG244283) and P0213T (MG244284). The 16S rRNA gene sequence similarity between strain P0211T and P0213T was 99.30%. There was also relatively high sequence similarities of strains P0211T and P0213T to closely related species *A. aestuariivivens* KCTC 52655T (97.18 and 97.53%, respectively) and *A. lipolytica* MCCC 1K03175T (96.98 and 97.25%, respectively). Phylogenetic analysis of the 16S rRNA gene sequences showed that the two isolates formed a highly significant cluster with one another. This cluster was clearly separated from the closest relative *A. aestuariivivens* and there was stable topology regardless of which tree reconstruction method was applied (Fig. 1). According to the MLPA analysis, strains P0211T and P0213T formed a tight group separated from the closely species within the genus *Alteromonas*, also confirming the results obtained from the phylogenetic analysis of the 16S rRNA gene sequence (Fig. 2). The same topologies were again obtained after ML and MP phylogenetic analysis (Fig. 2). Phylogenetic analysis of sequences of 16S rRNA gene and partial sequences of five housekeeping genes give support for the classification of two strains as novel species within the genus *Alteromonas*.

Draft genome sequencing of strain P0211T (accession number PEAX00000000) yielded a genome of 3,646,037 bp in length after assembly, producing 42 contigs, and a N50 value of 488,695. All contigs were larger than 261 bp and the largest was 902,613 bp. A total of 3398 genes were predicted of which 3316 were protein coding genes. Draft genome sequencing of strain P0213T (accession number PEAY00000000) produced a genome of 3,914,585 bp in length after assembly, producing 23 contigs, and a N50 value of 687,814. Contig size ranged from 261 bp to 1,340,369 bp. A total of 3,552 genes were predicted of which 3470 were protein coding genes. The G + C mol% contents of P0211T and P0213T strains were 45.8 and 45.3 mol%, respectively, which fall within the range given for species of the genus *Alteromonas*.

**Table 1**
Differential characteristics between strains P0211T, P0213T and the type strains of phylogenetically related *Alteromonas* species.
Strains: 1, *A. flava* P0211T (this study); 2, *A. facilis* P0213T (this study); 3, *A. aestuariivivens* KCTC 52655T (this study); 4, *A. lipolytica* MCCC 1K03175T (this study); 5, *A. macleodii* ATCC 27126T (this study); 6, *A. halophila* KCTC 22164T [4]; 7, *A. confluentis* KCTC 42603T [16]; 8, *A. genovensis* LMG 24078T [16,23].

| Characteristics | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| G + C content (mol%) | 45.8 | 45.3 | 51.1 | 48.4 | 44.6 | 47.5 | 48.6 | 44.5 |
| Reduction of nitrate | – | + | + | – | – | – | + | – |
| Citrate utilization | w | w | – | w | w | – | w | – |
| Hydrolysis of | | | | | | | | |
| Casein | – | – | + | + | + | – | + | + |
| Starch | + | + | – | – | + | + | + | w |
| Tween 80 | – | – | + | + | + | + | + | w |
| Oxidation of | | | | | | | | |
| D-Maltose | w | – | + | + | – | + | + | + |
| Sucrose | – | – | + | + | + | – | + | + |
| D-Salicin | – | – | + | | + | – | w | – |
| α-D-Glucose | w | – | + | w | + | + | + | – |
| D-Mannose | – | – | – | – | + | + | – | – |
| D-Fructose | – | w | – | – | – | + | + | + |
| D-Galactose | – | – | + | + | – | – | + | + |
| *myo*-Inositol | – | + | – | – | – | – | + | – |
| Enzyme activities | | | | | | | | |
| Catalase | + | + | + | – | + | + | + | + |
| Esterase (C4) | + | + | + | + | + | – | – | + |
| Lipase (C14) | – | – | – | – | + | + | – | w |
| Cystine arylamidase | – | + | + | + | + | – | – | + |
| Trypsin | + | + | – | + | – | – | – | w |
| α-Chymotrypsin | + | + | – | + | – | + | – | + |
| β-Glucuronidase | – | – | – | + | – | – | + | – |
| α-Glucosidase | – | – | – | + | + | – | – | NA |
| β-Glucosidase | – | – | – | + | – | – | + | NA |
| Acid from | | | | | | | | |
| D-Ribose | + | + | + | + | + | – | – | – |
| D-Xylose | – | – | + | – | + | – | + | – |
| D-Fructose | – | – | – | – | + | + | – | + |
| Cellobiose | – | – | + | + | + | – | + | – |
| Lactose | – | – | – | + | + | – | + | – |
| Melibiose | – | – | – | + | + | + | – | – |
| Trehalose | – | – | – | + | + | + | – | – |
| Melezitose | – | – | – | + | + | – | + | – |

+, Positive reaction; –, negative reaction; w, weakly positive reaction; NA, no data available.
All strains are positive for oxidase, alkaline phosphatase, esterase lipase (C8) and leucine arylamidase activities. All strains are negative for indole production; acid production from D-mannose, inositol and D-sorbitol; and activity of *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase.

According to the data obtained, the ANIb and ANIm between strains P0211T and P0213T were 72.58 and 84.20%, respectively; the ANIb and ANIm between the two strains with the other species tested were lower than 86% (Table 2). The species cut-off value for ANIb was selected as recommended at 95–96% [18]. The DDH between strains P0211T and P0213T was 18.6%. The DDH comparison with the draft genome of the two strains yielded low percentages (<26%) with all the species tested (Table 2). The species cut-off value for DDH was 70% [13]. The POCP values of strains P0211T and P0213T with the type strain of the genus *Alteromonas*, *A. macleodii* ATCC 27126T, were 59.05 and 56.95%, respectively. A POCP value of 50% has been proposed as a genus boundary for prokaryotic lineages [17]. Comparing strains P0211T and P0213T, the identities of *dnaK*, *sucC*, *rpoB*, *gyrB*, and *rpoD* were 85, 77, 85, 77 and 80%, respectively. In addition, from comparing the two strains with other *Alteromonas* species, the identities of five housekeeping genes belonging to strains P0211T were lower than 80%, and the

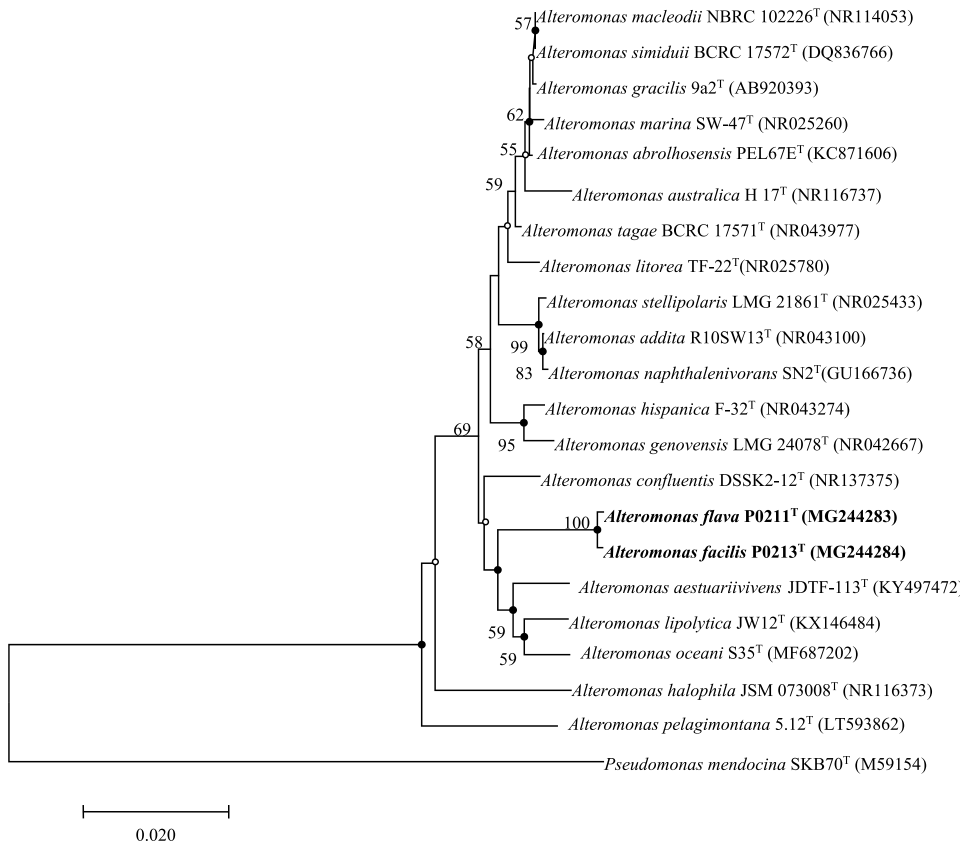


**Fig. 1.** Phylogenetic tree constructed with 16S rRNA gene by the NJ algorithm, showing the position of strains P0211$^{T}$ and P0213$^{T}$ among related taxa. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at the nodes. Filled circles indicate that the corresponding nodes were also recovered in ML and MP algorithm. Open circles indicate that the corresponding nodes were also recovered in either the ML or MP algorithm. Bar, 0.020 substitutions per nucleotide position.

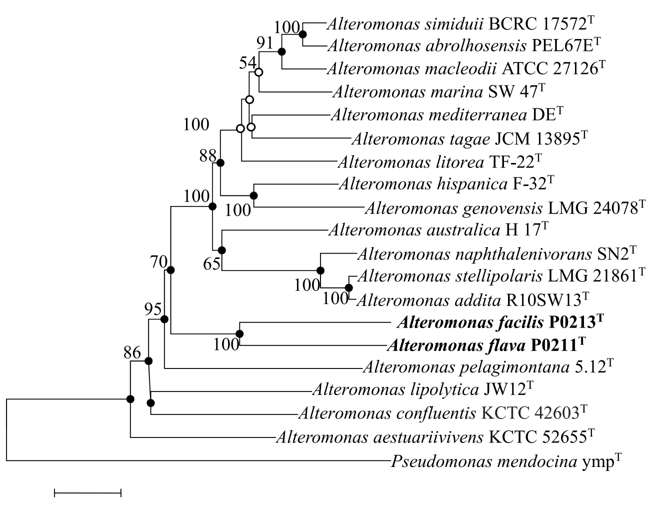


**Fig. 2.** Phylogenetic reconstruction based on the concatenated sequences of *dnaK*, *sucC*, *rpoB*, *gyrB*, and *rpoD* genes by the NJ algorithm, showing the position of strains P0211$^{T}$ and P0213$^{T}$. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at the nodes. Filled circles indicate that the corresponding nodes were also recovered in ML and MP algorithm. Open circles indicate that the corresponding nodes were also recovered in either the ML or MP algorithm. Bar, 0.050 substitutions per nucleotide position.

identities of five house-keeping genes belonging to strain P0213$^{T}$ were lower than 81% (Table S3). The five house-keeping genes revealed a threshold value of 98.9% that could be considered as the species cut-off value for the delineation of *Alteromonas* spp. [9,10]. These values supported the classification of the two isolates within the genus *Alteromonas*, representing two as yet undescribed species of this genus.

**Table 2**
ANIb, ANIm and DDH value (%) of strains P0211$^{T}$ and P0213$^{T}$ with their phylogenetically related species.
Strains: 1, *A. flava* P0211$^{T}$; 2, *A. facilis* P0213$^{T}$; 3, *A. aestuariivivens* KCTC 52655$^{T}$; 4, *A. lipolytica* JW12$^{T}$; 5, *A. confluentis* KCTC 42603$^{T}$; 6, *A. abrolhosensis* PEL67E$^{T}$; 7, *A. addita* R10SW13$^{T}$; 8, *A. australica* H 17$^{T}$; 9, *A. macleodii* ATCC 27126$^{T}$; 10, *A. marina* SW-47$^{T}$; 11, *A. mediterranea* DE$^{T}$; 12, *A. naphthalenivorans* SN2$^{T}$; 13, *A. pelagimontana* 5.12$^{T}$; 14, *A. stellipolaris* LMG 21861$^{T}$.

| Species name | ANIb | | ANIm | | DDH | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 1 | 2 | 1 | 2 |
| 1 | * | 72.64 | * | 84.20 | * | 18.6 |
| 2 | 72.58 | * | 84.20 | * | 18.6 | * |
| 3 | 67.93 | 67.94 | 85.62 | 85.78 | 20.9 | 21.3 |
| 4 | 68.02 | 68.17 | 84.73 | 84.77 | 25.6 | 23 |
| 5 | 67.70 | 68.03 | 84.82 | 85.28 | 21.4 | 21.4 |
| 6 | 68.27 | 68.50 | 85.37 | 85.20 | 21.6 | 20.5 |
| 7 | 68.05 | 68.60 | 84.54 | 85.02 | 23.4 | 21.6 |
| 8 | 68.11 | 68.29 | 85.61 | 84.82 | 21.7 | 22.4 |
| 9 | 68.24 | 68.70 | 85.39 | 84.85 | 21.7 | 22.5 |
| 10 | 68.32 | 68.60 | 85.52 | 84.96 | 21.7 | 20.6 |
| 11 | 68.27 | 68.48 | 85.56 | 85.57 | 22.1 | 21.2 |
| 12 | 68.07 | 68.48 | 85.49 | 85.77 | 23.0 | 23.7 |
| 13 | 68.89 | 70.35 | 85.01 | 85.94 | 25.2 | 23.2 |
| 14 | 67.97 | 68.43 | 84.60 | 84.69 | 23.1 | 21.2 |

The isolates were screened for copper tolerance, and genome analysis demonstrated the existence of genes associated with copper resistance, including *cutA*, *cutE*, *cusA*, *cusB*, *copD*, *cusR*, and *cpxA*/*cpxR* (Table S4). It has been reported previously that these genes confer resistance against high levels of copper, and a mutation in one or more of these genes results in an increased copper

**Table 3**
Cellular fatty acid composition (%) of strains P0211$^T$, P0213$^T$ and related species. Strains: 1, *A. flava* P0211$^T$; 2, *A. facilis* P0213$^T$; 3, *A.aestuariivivens* KCTC 52655$^T$; 4, *A. lipolytica* MCCC 1K03175$^T$; 5, *A. macleodii* ATCC 27126$^T$. All data listed in the table are from this study.

| Fatty acid | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Straight-chain fatty acids | | | | | |
| $C_{12:0}$ | 2.70 | 3.76 | 1.65 | 2.50 | 3.55 |
| $C_{14:0}$ | 3.02 | 5.01 | 3.10 | 3.20 | 4.28 |
| $C_{15:0}$ | 4.43 | 4.34 | 2.03 | 1.38 | 1.37 |
| $C_{16:0}$ | **24.26** | **19.78** | **36.20** | **23.54** | **25.01** |
| $C_{17:0}$ | 2.75 | 1.36 | 3.30 | 1.21 | 2.08 |
| $C_{18:0}$ | – | – | 8.90 | 1.56 | 1.21 |
| Unsaturated fatty acids | | | | | |
| $C_{15:1}\omega 8c$ | 3.45 | 3.50 | – | – | 1.07 |
| $C_{17:1}\omega 8c$ | 4.75 | 4.00 | 4.20 | 3.14 | 3.32 |
| $C_{18:1}\omega 7c$ | 6.42 | 5.53 | **11.50** | 7.27 | **10.33** |
| Branched fatty acids | | | | | |
| iso-$C_{16:0}$ | 1.28 | 1.28 | – | 2.24 | – |
| Hydroxy fatty acids | | | | | |
| $C_{10:0}$ 3-OH | – | 0.18 | 1.17 | 0.34 | 1.55 |
| $C_{11:0}$ 3-OH | 1.00 | – | 0.40 | – | – |
| $C_{12:0}$ 3-OH | 4.05 | 3.95 | 1.23 | 1.81 | 1.53 |
| $C_{12:1}$ 3-OH | – | – | – | 2.17 | – |
| Summed feature[a] | | | | | |
| 2 | – | 1.25 | 2.40 | 2.82 | 4.20 |
| 3 | **33.45** | **37.02** | **15.3** | **42.63** | **31.21** |

–, Trace quantities (<1%).
Bold type indicates major components (>10 %).
[a] Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed features 2 consisted of iso-$C_{16:1}$ I and/or $C_{14:0}$ 3-OH and/or $C_{12:0ALDE}$, and Summed features 3 consisted of $C_{16:1}\omega 7c$ and/or iso-$C_{15:0}$ 2-OH.

sensitivity. Moreover, we found that these copper resistance genes, including *cut*, *cus*, *cop*, and *cpxA*/*cpxR*, are also widely present in other *Alteromonas* genomes (Table S4).

The major fatty acids (relative amount >10%) found in the two strains were identified as $C_{16:1}\omega 7c$/iso-$C_{15:0}$ 2-OH and $C_{16:0}$. As expected, the isolates and reference strains shared similar fatty acid profiles, but the relative amount of some fatty acids in the two strains was different compared with the reference strains (Table 3). The major polar lipids of strains P0211$^T$ and P0213$^T$ were phosphatidylethanolamine (PE) and phosphatidylglycerol (PG), which was consistent with reference strains (Fig. S2).

Based on the polyphasic analyses including phenotypic, chemotaxonomic, and genomic characterizations presented in this study, we propose to classify the tested isolates as two new species, *Alteromonas flava* sp. nov. (type strain P0211$^T$) and *Alteromonas facilis* sp. nov. (type strain P0213$^T$).

## *Description of* A. flava *sp. nov.*

***A. flava*** [*fla'va*. L. fem. adj. flava, yellow, the color of its colonies].

Cells are Gram-negative, strictly aerobic, motile rods with one polar flagellum (0.4–0.8 μm wide and 0.8–3.6 μm long). Colonies are yellow-colored, circular, and smooth on MA. Growth occurs at 15–45 °C (optimum, 37 °C), pH 6.5–8.5 (optimum, pH 8.0–8.5), and 1.0–7.0% (w/v) NaCl (optimum, 2.0%). Oxidase- and catalase-positive. Hydrolyzes Tweens 20, 40, and 60, and starch, but not agar, casein, cellulose, and alginate. Nitrate reduction is negative. Production of indole and $H_2S$ is negative. Tests positive for gelatinase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, and α-chymotrypsin, and but tests negative for urease, ornithine decarboxylase, lysine decarboxylase, arginine dihydrolase, lipase (C14), valine arylamidase, cystine arylamidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-fucosidase, and α-mannosidase. D-cellobiose, gentiobiose, formic acid, D-fructose-6-$PO_4$, L-alanine, D-galacturonic acid, glucuronamide, α-keto-glutaric acid, acetoacetic acid, and acetic acid are oxidized, and D-maltose, D-turanose, D-glucuronic acid, and α-D-glucose are weakly oxidized in Biolog GEN III microplates. Acid is produced from D-ribose, aesculin, D-tagatose, and potassium 5-ketogluconate in the API 50 CHB fermentation kits. The major fatty acids are $C_{16:1}\omega 7c$/iso-$C_{15:0}$ 2-OH and $C_{16:0}$. The major polar lipids are phosphatidylethanolamine and phosphatidylglycerol. The DNA G + C of the type strain is 45.8 mol%.

The type strain, P0211$^T$ (= KCTC 62078$^T$ = MCCC 1H00242$^T$, taxonnumber TA00625) was isolated from a sea cucumber culture pond located in Rongcheng, Shandong province, China.

## *Description of* A. facilis *sp. nov.*

***A. facilis*** [*fa'ci.lis*. L. fem. adj. facilis ready, quick, referring to the ease of cultivation of the organism].

Cells are Gram-negative, facultatively anaerobic, motile rods with one polar flagellum (0.3–0.6 μm wide and 1.0–4.2 μm long). Colonies are beige-colored, circular and smooth on MA. Growth occurs at 15–45 °C (optimum, 37 °C), pH 6.5–8.5 (optimum, pH 8.0–8.5), and 1.0–7.0% (w/v) NaCl (optimum, 2.0%). Oxidase- and catalase-positive. Hydrolyzes Tweens 20, 40 and 60, and starch, but not agar, casein, cellulose and alginate. Nitrate is reduced to nitrite. Production of indole and $H_2S$ is negative. Tests positive for gelatinase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, cystine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, and α-chymotrypsin, and but tests negative for urease, ornithine decarboxylase, lysine decarboxylase, arginine dihydrolase, lipase (C14), valine arylamidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-fucosidase, and α-mannosidase. D-turanose, *myo*-inositol, D-fructose-6-$PO_4$, glucuronamide, and α-keto-glutaric acid are oxidized, and D-fructose, D-galacturonic acid, and D-glucuronic acid are weakly oxidized in Biolog GEN III microplates. Acid is produced from D-ribose, aesculin, D-tagatose, and potassium 5-ketogluconate in the API 50 CHB fermentation kits. The major fatty acids are $C_{16:1}\omega 7c$/iso-$C_{15:0}$ 2-OH and $C_{16:0}$. The major polar lipids are phosphatidylethanolamine and phosphatidylglycerol. The DNA G + C of the type strain is and 45.3 mol%.

The type strain, P0213$^T$ (= KCTC 62079$^T$ = MCCC 1H00243$^T$, taxonnumber TA00626) was isolated from a sea cucumber culture pond located in Rongcheng, Shandong province, China.

## *Emended description of the genus* Alteromonas

The description of the genus *Alteromonas* is based on that given previously by Baumann et al. [2]. Species of the genus are strictly aerobic or facultatively anaerobic.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.syapm.2018.11.006.

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