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Physiological, Ecological, and Phylogenetic Characterization of *Stappia*, a Marine CO-Oxidizing Bacterial Genus▽

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Bacteria play a major role in marine CO cycling, yet very little is known about the microbes involved. Thirteen CO-oxidizing *Stappia* isolates obtained from existing cultures, macroalgae, or surf samples representing geographically and ecologically diverse habitats were characterized using biochemical, physiological, and phylogenetic approaches. All isolates were aerobic chemoorganotrophs that oxidized CO at elevated (1,000 ppm) and ambient-to-subambient concentrations (<0.3 ppm). All contained the form I (OMP) *coxL* gene for aerobic CO dehydrogenase and also the form II (BMS) putative *coxL* gene. In addition, some strains possessed *cbbL*, the large subunit gene for ribulose-1,5-bisphosphate carboxylase/oxygenase, suggesting the possibility of lithotrophic or mixotrophic metabolism. All isolates used a wide range of sugars, organic acids, amino acids, and aromatics for growth and grew at salinities from 5 to 45 ppt. All but one isolate denitrified or respired nitrate. Phylogenetic analyses based on 16S rRNA gene sequences indicated that several isolates could not be distinguished from *Stappia aggregata* and contributed to a widely distributed species complex. Four isolates (of strains GA15, HI, MIO, and M4) were phylogenetically distinct from validly described *Stappia* species and closely related genera (e.g., *Ahrensia*, *Pannonibacter*, *Pseudovibrio*, and *Roseibium*). Substrate utilization profiles, enzymatic activity, and membrane lipid composition further distinguished these isolates and supported their designations as new *Stappia* species. The observed metabolic versatility of *Stappia* likely accounts for its cosmopolitan distribution and its ability to contribute to CO cycling as well as other important biogeochemical cycles.

The marine genus *Stappia* encompasses four phylogenetically distinct chemoorganotrophic species in the α-2 subgroup of the *Proteobacteria* (9, 27, 38). *Stappia stellulata* and *Stappia aggregata* were originally isolated from coastal marine water column and sediment samples, assigned to the genus *Agrobacterium*, and subsequently transferred to the genus *Stappia* (1, 29, 38). *Stappia alba* (27), *Stappia marina* (9), and various *Stappia*-like isolates have since been obtained from numerous widely distributed sources, including warm temperate surface and permanently cold deep-sea waters, sediments, phytoplankton, macroalgae, invertebrates, and salt marshes (2, 3, 5, 9, 13, 17, 26, 27, 33; Donachie et al., unpublished data). In addition, the presence of *Stappia* or *Stappia*-like taxa in a similar range of habitats has also been inferred from cultivation-independent analyses (3, 26; Donachie et al., unpublished).

Neither the original description (29) nor the subsequent work by Uchino et al. (38) addressed the geographic distribution of *Stappia* or its physiological and ecological attributes. Results of subsequent studies have shown that *Stappia*-like isolates can account for a significant percentage of cultivable α-*Proteobacteria* containing dioxygenase genes (3). *Stappia* or *Stappia*-like isolates have been reported to produce sodium channel-blocking proteins (5, 34) and a rhizobactin-like siderophore (20). *Stappia* strains are thus functionally versatile, occupy several ecological niches, and participate in biogeochemical cycles and processes that are important on microscales to global scales (e.g., CO oxidation and denitrification).

To date, all *Stappia* strains have been obtained from heterotrophic enrichments. Accordingly, the genus has been described as chemoorganotrophic (38). Some strains, however, may function as facultative lithotrophs. All *Stappia* strains examined to date oxidize carbon monoxide and possess the form I (OMP) *coxL* gene encoding the large subunit of carbon monoxide dehydrogenase (CODH) (9, 13). Some also contain a gene for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) (*cbbL*) and may be able to couple CO utilization to CO_2 fixation (13).

In spite of their ubiquity, relatively little is known about the phylogenetic and physiological relationships among *Stappia* isolates or the responses to environmental variables that may affect their distributions and activities. We have enriched and isolated *Stappia* spp. from geographically diverse habitats and characterized novel strains using biochemical, physiological, and molecular approaches. We have also determined the extent to which isolates oxidize CO at elevated and near ambient concentrations. The results collectively support the designation of four new species: *Stappia conradae*, *Stappia meyerae*, *Stappia carboxidovorans*, and *Stappia kahanamokuae*.

MATERIALS AND METHODS

Culture sources and isolation. *Stappia aggregata* and *Stappia* sp. strains CV902-700 and CV812-530 were obtained from the Damariscotta River, Maine (2). *Stappia* sp. strain MIO was obtained from a marine methanotrophic consortium (gift of M. Takeuchi, National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan). *S. stellulata* was obtained from the Deutsche

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TABLE 1. Substrate utilization profiles for *S. aggregata*, *S. stellulata*, and strains MIO, HI, BrT4, GA15, M4, and M8[a]

Characteristic	*S. stellulata*	*S. aggregata*	MIO	HI	BrT4	GA15	M4	M8
Average cell size (μm) (length × width)		1.9 × 0.8	1.8 × 0.7	2.1 × 0.7	2.4 × 0.8	2.0 × 0.8	1.6 × 0.8	2.4 × 0.8
Sodium requirement	+	+	+	+	+	+	+	+
CO V_{max} (nmol [mg protein]$^{-1}$ h^{-1})	NT		270	350	65	310	400	510
Enzymatic activities:								
Nitrate respiration	−	−*	+	−	−	−	−	−
Denitrification	+	+*	−	+	−	+	+	+
Indole production	−	NT	−	−	−	−	−	−
Fermentation	−	−*	W	−	−	W	W	W
Arginine dihydrolase	−	NT	−	−	−	−	−	−
Urease	−	NT	+	+	+	+	+	+
Esculine hydrolysis	−	NT	+	+	+	+	+	+
Gelatinase	−	NT	−	+	−	−	−	−
β-Galactosidase	−	NT	+	+	+	+	+	+
Growth substrates:								
Isophthalate	−	W	−	−	−	−	−	W
Terephthalate	−	−	+	+	+	+	I	W
4-Hydroxybenzoate	I	W	−	−	−	−	I	+
Phthalate	W	−	W	−	−	+	−	−
Methanol	−	I	−	−	−	−	−	−
Ethanol	−	I	NT	−	−	NT	−	−
Isopropanol	−	I	−	−	−	−	−	−
Acetone	W	I	−	−	−	−	−	−
Formate	−	−	W	−	−	−	−	−
Propionate	+	+	+	+	−	+	+	+
Glycolate	W	−	NT	+	+	NT	W+	W+
Malonate	+	+	W	−	W	+	+	+
Tartrate	I	−	W	W	+	−	−	−
Lactate	W	+	+	+	+	+	+	W+
Gluconate	−	W	+	+	−	+	+	+
Glycerol	NT	+	W	+	−	+	+	+
Galactose	W	+	+	+	NT	+	+	+
Mannose	W	+	+	+	+	+	+	+
Ribose	+	+	W	+	+	+	+	+
Lactose	+	W	+	+	W	+	+	W
Sucrose	+	+	NT	+	+	NT	+	+
Glucuronate	W	+	+	+	+	+	+	+
Galacturonate	NT	+	NT	+	NT	NT	+	W
Mannitol	−	+	+	+	+	+	+	+
Glycine	NT	I	−	−	−	−	−	−
Alanine	+	+	+	−	−	+	+	+
Valine	+	+	W	+	+	+	+	+
Glutamate	+	+	+	−	−	+	+	+
Aspartate	+	W	W	+	R	+	W+	W+
Serine	−	−	NT	−	−	NT	I	+
Phenylalanine	+	I	−	−	W	W	−	−
Benzoate	I	I	−	−	−	−	NT	NT
Methylamine	W	I	−	−	−	−	−	I
Dimethylamine	W	I	−	−	−	−	−	−
Trimethylamine	W	I	I	−	−	−	−	−
Betaine	+	W	W	+	+	+	+	+
G+C content (%)	59*	59*	58.3	60.9	56.8	57.9	57.4	57.8

[a] +, growth; −, no growth; W, weak growth; I, substrate was inhibitory; NT, substrate was not tested; *, data obtained from Uchino et al. (40); V_{max}, maximum rate of metabolism.

Samlung für Mikroorganizmen und Zellkulturen (Braunschweig, Germany). *Stappia* sp. strains SE 09 and SE11 were obtained from the Duplin River, Georgia (A. Buchan, University of Tennessee) (3). *Stappia* strains M4 and M8 were obtained from enrichments based on marine macroalgae as previously described (13).

Additional *Stappia* strains were obtained from seawater and macroalgae by incubating 20 to 100 ml basal salts medium (PYE) (20) containing 0.01 to 0.05% yeast extract and 25 mM pyruvate with 1 ml of surf water samples from Ka Lae, Hawaii, or 1 to 5 g fresh weight of macroalgae: *Gracilaria* sp., *Ulva* sp., *Caulerpa* sp. or an unidentified, tube-shaped green alga, *Ascophyllum nodosum*, or *Ulva lactuca* (the latter was from the Damariscotta River, Walpole, ME). CO was added at 100- to 1,000-ppm concentrations to enrichment flask headspaces, and uptake was monitored using gas chromatography (11). Enrichments positive for CO oxidation were diluted serially and plated onto PYE prepared with artificial seawater (ASW) (MPYE). Colonies were selected arbitrarily and transferred to liquid MPYE and grown to stationary phase. Cultures that oxidized CO were purified by plating serial dilutions on solid MPYE and transferred to liquid medium.

Morphological, physiological, and biochemical characterization. Growth-supporting substrates were examined using a basal salts medium supplemented with 0.05% yeast extract (21) and various carbon substrates. Growth was assayed spectrophotometrically after 48 h and compared to controls containing 0.05% yeast extract only. Selected biochemical traits were assayed using API 20 NE strips according to the manufacturer's instructions (BioMerieux, Inc., France). Catalase and oxidase tests and Gram staining were performed using standard methods (4). Major membrane lipids of *S. aggregata* and strains BrT4, GA15, HI,

M4, M8, and MIO were obtained from Microbial ID, Inc. (Newark, DE). A Zeiss Axioscope fitted with a Neofluar 100× objective and an AxioCam MR digital camera was used to obtain images of logarithmically growing cells for size estimates and to determine motility.

G+C determination. Nucleotides were hydrolyzed enzymatically from RNA-free genomic DNA, then dephosphorylated to produce nucleosides. Nucleosides were separated by high-pressure liquid chromatography using a C_{18} column and an isocratic mobile phase of ammonium phosphate and methanol and detected by UV absorption (254 nm). Reference standards include genomic DNA from *Escherichia coli*, *Burkholderia xenovorans*, and *Mycobacterium smegmatis* for which percent G+C contents are known from genomic sequences.

CO utilization. To determine maximum CO uptake velocities, *Stappia* isolates were grown to stationary phase in MPYE, harvested by centrifugation, washed with buffered ASW, and resuspended to an optical density (OD) of 0.5 in buffered ASW containing mineral salts (21) and 0.005% yeast extract. Five-milliliter cell suspensions for each isolate were transferred to triplicate 160-cm^3 serum bottles. CO was added to bottle headspaces (1,000 ppm), and cultures were incubated with shaking (200 rpm) at 30°C. CO uptake was monitored at intervals by gas chromatography. Maximum uptake velocities were expressed per milligram of protein after the measurement of culture protein contents using the bicinchoninic acid assay kit (Pierce, Inc.).

The ability of *Stappia* isolates to consume CO at ambient concentrations was assessed using triplicate 10-ml cultures of strains M4, GA15, MIO, and HI that were grown to stationary phase and transferred to 160-cm^3 serum bottles. The cultures were sealed and incubated at 30°C with rotary shaking (200 rpm) and initial CO concentrations of about 2 ppm. Headspace subsamples were obtained at intervals by needle and syringe for analysis using an RGA-3 reduced gas analyzer (Trace Analytical, Inc.).

Heterotrophic substrate effects on *S. aggregata* CO uptake. *S. aggregata* grown overnight on MPYE was harvested by centrifugation, washed with buffered ASW, and resuspended in basal salts with 0.005% yeast extract. Six sets of sealed triplicate 160-cm^3 serum bottles containing 4.5 ml of marine basal salts (21) with glucose concentrations of 0, 0.5, 2.5, 5.0, 10, or 20 mM were inoculated by syringe and needle with 0.5 ml of washed culture. CO was added to the bottle headspaces (1,000 ppm), and absorbance (Abs; optical density at 600 nm [OD_{600}]) and headspace CO concentrations were monitored at suitable intervals. CO uptake rates were determined from analyses of CO concentrations over time using KaleidaGraph software (version 4.0.5; Synergy Software, Inc.) as previously described by Hardy and King (6, 12). Cell biomass was estimated from absorbance using the following empirical expression after correcting for differences in absorbance at 600 and 660 nm (16):

$$\mu g \text{ dry weight } ml^{-1} = (364.74)(Abs600\text{ nm}) + (6.7)(Abs600\text{ nm}).$$

Growth response to varied salinities. Growth responses to salinities ranging from 0 to 45 ppt were determined for strains GA15, HI, M4, and MIO by growing the isolates in 250-ml Erlenmeyer flasks containing 50 ml basal salts medium (20) in artificial seawater supplemented with 0.05% yeast extract; salinities were adjusted to 5, 15, 25, 35, and 45 ppt. Nonmarine media were created using deionized water. A sodium-free medium was created by using nonsodium salts (from 21) and deionized water. Filter-sterilized glucose (25 mM) was added to sterile basal medium. All flasks were inoculated with fresh cultures (<24 h) grown on MPYE. Cultures used for sodium-free medium were harvested by centrifugation (1 min; 13,000 rpm), washed in buffered ASW, and resuspended in sodium-free medium prior to inoculation. Culture absorbance (OD_{600}) was monitored immediately following inoculation and at intervals thereafter. Specific growth rate constants were determined by fitting absorbance data to a modified Gompertz equation (40) using KaleidaGraph software (version 4.0.5; Synergy Software, Inc.) to obtain the fitting parameters. All assays were conducted in triplicate.

DNA extraction, PCR, sequencing, and analysis. Genomic extracts were obtained using UltraClean microbial DNA isolation kits (MO BIO Laboratories, Inc., Carlsbad, CA) as we have previously described (13). 16S rRNA genes were amplified using primers 27f and 1492r (18, 30). PCRs were carried out using 50-μl volumes (13). A 492- to 495-bp *cbbL* fragment was amplified and sequenced using primers K2 and V2 as previously described by Nanba et al. (22). OMP *coxL* genes (form I; about 1,260 bp) and putative *coxL* genes (BMS, form II; about 1,260 bp) were also amplified from the isolates and sequenced as we have described previously (13). PCR products of the correct size were purified using a MO BIO UltraClean PCR cleanup kit (MO BIO Laboratories, Inc., Carlsbad, CA) and then sequenced bidirectionally with an ABI model 377 sequencer at the University of Maine DNA Sequencing Facility using the amplification primers (Orono, ME).

TABLE 2. Fatty acid composition (%) for *S. aggregata* and strains MIO, HI, BrT4, GA15, M4, and M8

Fatty acid	*S. aggregata*	MIO	HI	BrT4	GA15	M4	M8
17:1ω7c				0.6	0.4		
18:1ω5c		0.2	0.2	0.3	0.2	0.2	
18:1ω7c	71.7	69.4	72.6	80.7	71.7	83.3	86.3
20:1ω9c		1.0					
20:4ω6,9,12,15c			0.4				
11-methyl 18:1ω7c	8.6	7.7	4.5	0.6	9.6	2.2	1.0
12:0				0.2			
14:0		0.20		0.5			
16:0	1.3	3.0	0.7	2.5	0.7	0.5	1.4
17:0	1.3	0.1	0.4	0.3	1.8	0.2	0.5
18:0	8.7	10.3	10.5	5.5	9.2	7.9	7.8
19:0	1.4		0.4		1.4	0.2	
20:0	1.6	3.4	7.1	0.3	1.6	1.4	1.7
18:0-3OH	0.9	0.9	0.6	1.1	0.4	0.8	
18:0iso		0.9		0.7			
20:0iso		0.4					

Phylogenetic analysis. 16S rRNA gene sequences for *Stappia* isolates and for various phylogenetic neighbors were obtained from the Ribosomal Database Project (www.rdp.cme.msu.edu/). Alignments were imported into ClustalX and further aligned manually as necessary. Aligned sequences were analyzed using maximum parsimony and distance (neighbor joining) algorithms as implemented in PAUP (version 4.0b; Sinauer Associates, Inc., Sunderland, MA). Maximum likelihood analyses were implemented using PHYML (http://atgc.lirmm.fr/phyml/) with an HKY model for base substitution and 100 bootstrap replicates.

BOX PCR. Genomic extracts were amplified using the BOXAR1 primer (19) in 50-μl PCRs containing recommended concentrations of deoxynucleoside triphosphates, buffers, Mg^{2+}, 1.25 U MasterTaq DNA polymerase (Brinkmann, Inc., Westbury, NY), dimethyl sulfoxide (2.5 μl), and the template. Amplification conditions were as previously described by Louws et al. (19). PCR products were electrophoresed at 70 V on a 1.25% agarose gel for 4 h at 4°C and visualized with GelRed (Biotium, Inc., Hayward, CA). Molecular weights of fragments were determined using Kodak Imaging software. A similarity index was created by dividing two times the number of shared fragments by the total number of fragments of a given pair of strains.

RESULTS

Morphological, physiological, and biochemical characterization. All strains were gram negative, nonsporing, motile rods with average dimensions of about 2 μm by 0.8 μm (length times width) (Table 1). All strains form irregular to star-shaped aggregates in liquid culture. Strain M4 also produced irregular forms in older cultures, including cells fused into a large spherical aggregate and cells that ballooned centrally or terminally. All strains formed circular, entire, smooth, slightly convex colonies with a light tan color on MPYE agar.

All strains grew on acetate, fumarate, citrate, succinate, pyruvate, β-hydroxybutyrate, malate, glucose, fructose, maltose, and proline. Strain M8 also grew on 4-hydroxybenzoate (4-HBA), which supported weak growth by *S. aggregata* and inhibited the growth of *S. stellulata* and strain M4 (Table 1). A purple metabolite accumulated transiently during the incubation of *S. aggregata* and *S. stellulata* with 4-HBA, indicating at least partial uncoupling of reactions in 4-HBA transformation (not shown). Terephthalate supported growth by some isolates (e.g., those of strains GA15, HI, and MIO), but inhibited the growth of strain M4. Isophthalate supported only weak or no growth, while phthalate supported growth by only strain GA15. None of the isolates grew on methylamines, methanol, isopro-

TABLE 3. Growth rate constants versus salinity

Salinity (ppt)	Growth rate (h^{-1}) of strain[a]:			
	HI	MIO	M4	GA15
5	0.134 (0.004)	0.136 (0.011)	0.273 (0.016)	0.224 (0.005)
15	0.176 (0.023)	0.178 (0.006)	0.330 (0.006)	0.231 (0.001)
25	0.221 (0.019)	0.164 (0.006)	0.336 (0.006)	0.218 (0.004)
35	0.229 (0.006)	0.148 (0.006)	0.265 (0.017)	0.201 (0.003)
45	0.256 (0.009)	0.097 (0.005)	0.220 (0.008)	0.159 (0.006)

[a] Results are averages of triplicate growth rate constants ± 1 standard error for growth rate constants and parts per thousand for salinity. Strains were grown in MPYE.

panol, acetone, or glycine, which proved inhibitory for *S. aggregata* (Table 1).

API test results were identical for strains GA15, M4, and M8 (Table 1). Strain MIO differed from the others in its ability to respire nitrate but not denitrify; strain BrT4 neither respired nitrate nor denitrified; strain HI differed in its ability to hydrolyze gelatin (Table 1). All strains were urease, β-glucosidase, and β-galactosidase positive; all strains were negative for indole production and arginine dihydrolase. Only weak evidence of fermentation was observed for strains GA15, M4, and M8.

All isolate membrane lipids were dominated by the fatty acid 18:1ω7c, followed by 18:0 and 11-methyl,18:1ω7c (Table 2). Similarity indices did not provide definitive matches with isolates in the existing Microbial ID database, but did differentiate strains M4 and M8 from *S. aggregata* and strains GA15, HI, and MIO (not shown). Individual isolates were differentiated from each other by the presence of 17:1ω7c in strains GA15 and BrT4 only, by the presence of 19:0 in all strains except MIO, BrT4, and M8, by the presence of 20:4ω6,9,12,15c in strain HI only, and by the presence of 14:0 and 18:0iso in strains BrT4 and MIO and by 20:0iso and 20:1ω9c in strain MIO only (Table 2).

G+C contents ranged from 56.8 to 61.2% (Table 1). G+C contents for *E. coli* strain DH5α, *Burkholderia xenovorans*, and *Mycobacterium smegmatis* were 50.8, 65.7, and 65.2%, respectively. Reported values from genomic sequences for several *E. coli* species and the latter two isolates were 50 to 51, 62.6, and 67.4%, respectively (www.ncbi.nlm.nih.gov/genomes/lproks.cgi).

Growth response to salinity. Strains GA15, HI, M4, and MIO grew exponentially when incubated in MPYE with salinities ranging from 5 to 45 ppt (Table 3). Analysis of variance revealed significant differences in growth rate constants as a function of strain and salinity ($P < 0.0001$). For strains GA15, M4, and MIO, maximum growth rate constants decreased at 45 ppt relative to growth rate constants at optimum salinities of 15, 15, and 25 ppt, respectively (Table 3). Strain HI exhibited the highest optimum salinity, 35 ppt. Growth rate constants were greater for strain M4 than for strains GA15 and MIO at all salinities and greater than the values for strain HI from 5 to 15 ppt. In contrast, values for strain HI were greatest at seawater and hypersaline salinities (35 to 45 ppt). All strains grew slowly (linearly) in a nonmarine, basal salts medium, with lower growth yields than those in marine media (not shown). All strains grew negligibly after inoculation into sodium-free media, even though residual sodium may have been present from the washing step (not shown).

Carbon monoxide utilization. All isolates oxidized carbon monoxide at concentrations of <1,000 ppm (0.1%), but higher concentrations proved inhibitory (Fig. 1). At concentrations of <1,000 ppm, CO uptake was consistent with Michaelis-Menten kinetics (Fig. 1), and apparent maximum uptake rate values ranged from about 270 to 510 nmol CO mg protein^{-1} h^{-1} (Table 1). CO was also consumed at ambient concentrations by strains GA15, HI, M4, and MIO (Fig. 2). Uptake rate constants were similar for the four strains, and within 17 h, all were able to reduce CO concentrations to values comparable to those reported for the marine water column. Continued incubation resulted in headspace concentrations of 30 to 40 ppb.

CO consumption by *S. aggregata* was partially inhibited by incubation with >0.5 mM exogenous glucose (Fig. 3). Con-

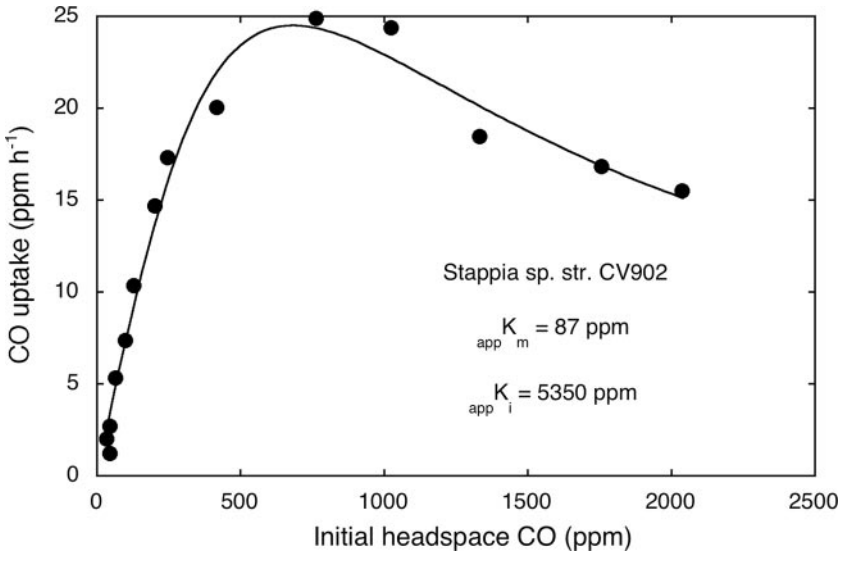

FIG. 1. CO uptake (ppm h^{-1}) as a function of initial headspace CO concentration for *Stappia* sp. strain KB902. Data are means of triplicates (±1 standard error).

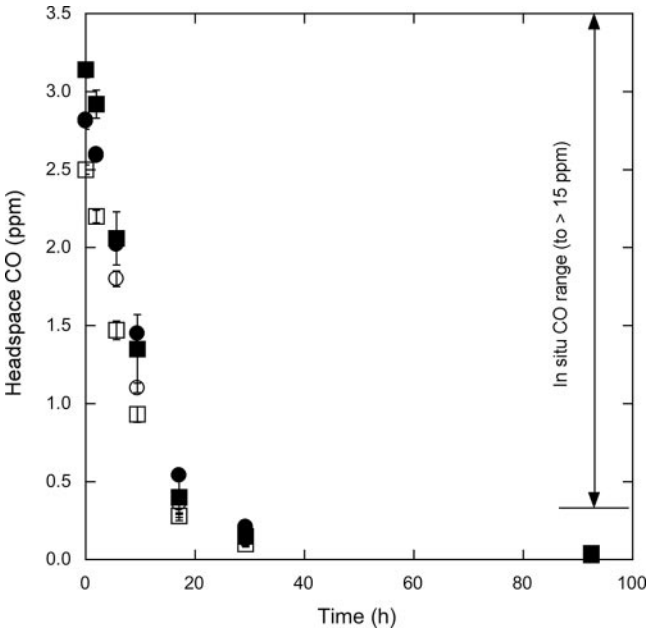

FIG. 2. Headspace CO concentrations in cultures of strains GA15 (●), HI (■), MIO (□), and M4 (○) at near ambient to subambient concentrations versus time (h). Error bars indicate standard errors.

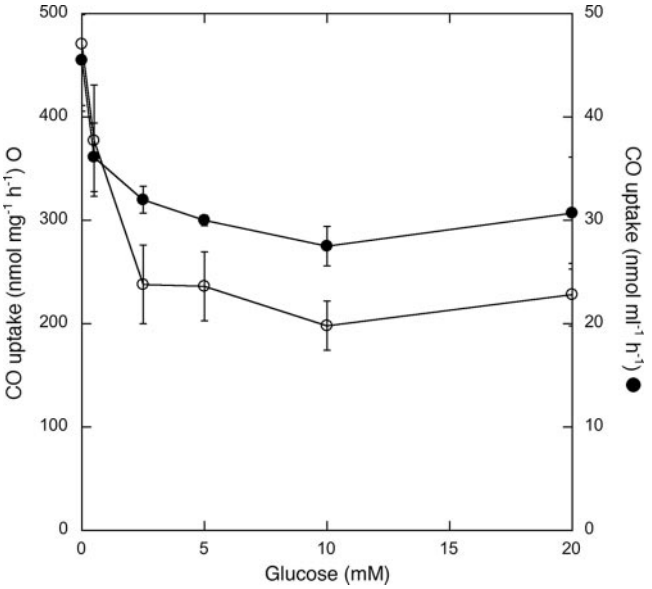

FIG. 3. CO oxidation rates (●, nmol [mgdw biomass]$^{-1}$ h^{-1}; ○, nmol [ml culture]$^{-1}$ h^{-1}) for triplicate cultures of *Stappia aggregata* as a function of the initial glucose concentration of the medium. Error bars indicate standard errors.

sumption rates decreased by up to 40% when expressed per unit volume of culture, even though *S. aggregata* density increased two- to threefold for glucose concentrations of ≥2 mM. When expressed per unit of cell biomass, increases in cell density were accompanied by decreases in uptake rates of up to 58% (Fig. 3).

Phylogenetic analyses. Phylogenetic trees derived from analyses of 16S rRNA gene sequences using maximum likelihood, maximum parsimony, and distance (neighbor-joining) models were topologically similar and characterized by the following consistent patterns (Fig. 4): (i) numerous isolate sequences and clone sequences from uncultured bacteria formed a cluster closely related to *S. aggregata*; (ii) 16S rRNA gene sequences from *S. aggregata* were clearly distinct from the sequence for *S. stellulata*; (iii) 16S rRNA gene sequences from strains GA15, HI, M4, and MIO were distinct from *S. aggregata* and *S. stellulata* as well as from *S. marina* and *S. alba* and other genera (e.g., *Pannonibacter*, *Pseudovibrio*, and *Roseibium*), which were not distinctly resolved from *Stappia* phylogenetically (Fig. 4). Other marine bacterial genera that form aggregates in liquid culture, e.g., *Ruegeria*, also appeared polyphyletic, but distinct from *Stappia* (Fig. 4). The phylogenetic distinctness of strains GA15, HI, M4, and MIO was consistent with 16S rRNA gene sequence similarities between these taxa and validly described *Stappia* spp., which ranged from 93.1 to 97.7%.

Form I (OMP) *coxL* sequences were obtained from all isolates examined in this study, while form II (putative, BMS) *coxL* sequences were obtained from all *Stappia* isolates but not from a Hawaiian *Photobacterium* isolate, *Mycobacterium marinum*, or a *Ruegeria* isolate (Fig. 5). Phylogenetic analysis revealed distinct OMP and BMS clades, within which sequences from *Stappia* were differentiated from those of other marine and nonmarine CO oxidizers (Fig. 5). The topology of the form I *Stappia coxL* cluster was generally similar to that of the form II cluster, with differences primarily in the location of branches for *Stappia* sp. strains MIO and M8. Within the form I and form II clades, sequences from several *Stappia* isolates were identical or nearly identical. OMP sequences from *S. aggregata*, *S. marina*, and *S. stellulata* were distinct from sequences for strains HI, MIO, and M4. An OMP *coxL* sequence from *M. marinum*, a marine actinobacterium, was distantly related to the proteobacterial sequences. In contrast, an OMP *coxL* sequence from a *Photobacterium* (γ-*Proteobacteria*) was remarkably similar to those of *Stappia* isolates (Fig. 5).

Partial sequences were obtained for the *cbbL* gene from a subset of *Stappia* isolates, including *S. aggregata* and strains BrT7, CV812, GA15, and MIO. PCR products were not obtained from *S. stellulata* or from strains BrC2, BrG2, BrT4, CV902, HI, M4, and M8. All of the *Stappia cbbL* sequences clustered with representatives of the form IC RuBisCO clade, based on a phylogenetic analysis (Fig. 6). Sequences from *S. aggregata* and strains BrT7, CV812, and MIO were nearly identical and clearly distinct phylogenetically from the strain GA15 sequence. *Stappia cbbL* sequences were not closely related to form IC sequences from other *Proteobacteria*, including several CO-oxidizing isolates.

BOX-PCR banding patterns were distinctly different for the various *Stappia* isolates examined and exhibited little similarity (Table 4). Similarity indices (S_{AB}) for pairwise comparisons ranged from 0.0 to 0.4, which indicated substantial differentiation among taxa (Table 3). S_{AB} values derived from comparisons of *S. aggregata* with all other strains, including *S. stellulata*, were ≤0.13; values for comparisons of all strains with *S. stellulata* were ≤0.36. In a number of instances, S_{AB} values were 0 due to the absence of shared bands. The greatest similarities were observed for comparisons of strains HI and M4 (S_{AB} = 0.4) and strains MIO and *S. stellulata* (S_{AB} = 0.36).

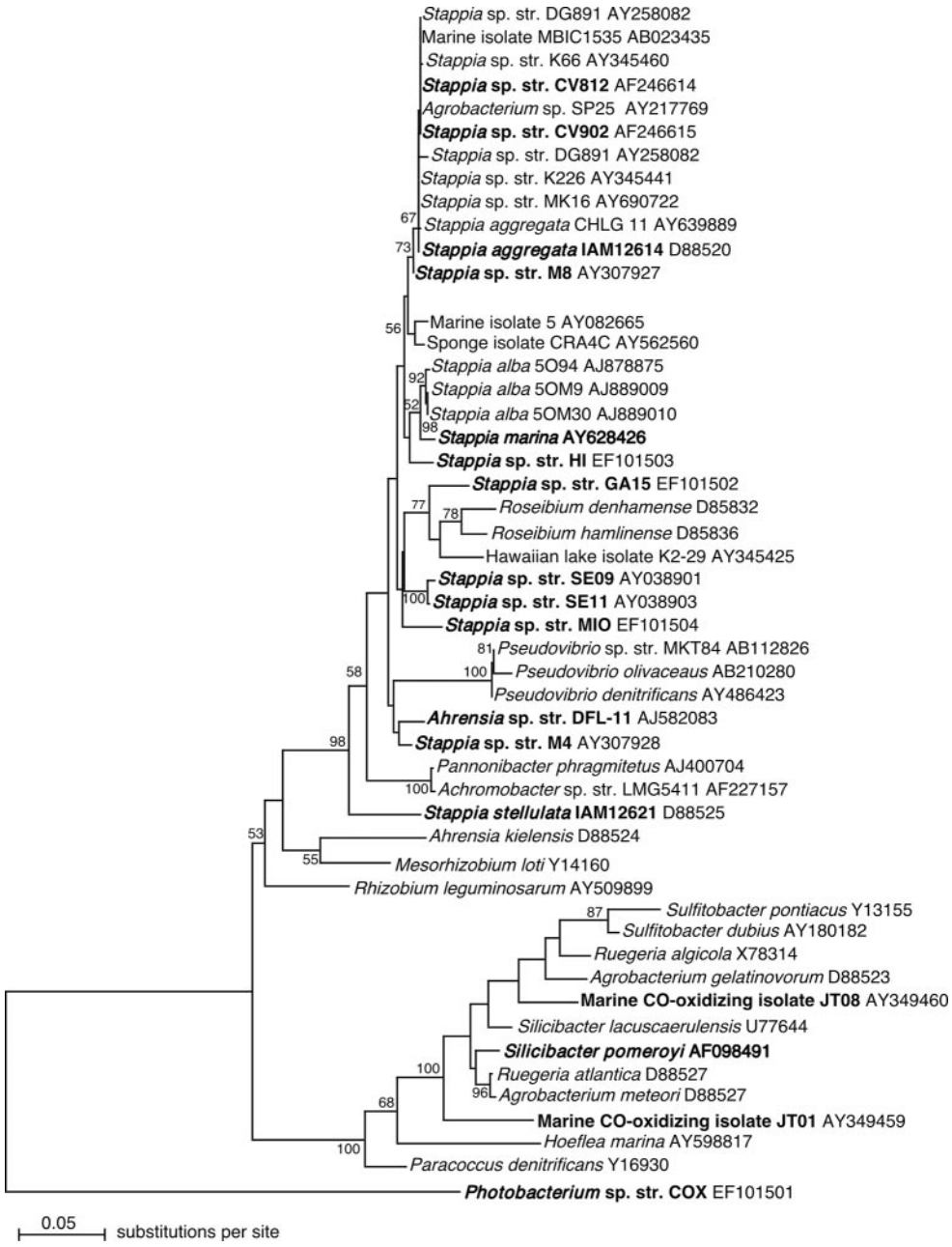

FIG. 4. Maximum likelihood analysis of partial 16S rRNA gene sequences (100 bootstrap replicates) implemented with phyml using an HYK correction. The phylogenetic tree was visualized with NJplot. Numbers at nodes indicate bootstrap support; values <70% are not shown. Known CO-oxidizing bacteria are indicated in bold. *Photobacterium* sp. strain (str.) COX was used as an outgroup.

DISCUSSION

Our results and those of others indicate that *Stappia* strains are readily isolated from many marine environments (1, 5, 9, 17, 27, 33). Though *Stappia* strains have been considered "marine" based on sodium requirements and weak growth in nonmarine media (1, 27, 38), three of four isolates in this study (strains GA15, MIO, and M4) exhibit growth optima in diluted seawater (Table 3). Only strain HI grows optimally in full-strength seawater (35 ppt). While strains GA15, MIO, and M4 occur in coastal environments, isolate growth optima do not reflect in situ salinity regimes since all are derived from systems with relatively constant salinities greater than 30 ppt. Although *S. stellulata* was originally described as an obligate halophile (1), salinity optima have not been reported for *S. aggregata* and *S. alba*. Thus, it is not clear to what extent adaptation to seawater varies within the genus or how *Stappia* compares in its salinity tolerance with other "marine" genera.

All *Stappia* isolates examined to date oxidize CO and contain form I *coxL* (Fig. 5 and Tables 1 and 3) (13). CO uptake capacity has not been reported for *S. alba* or *S. marina*, but *S. marina* possesses a form I CODH gene (9), a strong predictor of its physiological capacity. All *Stappia* isolates examined to

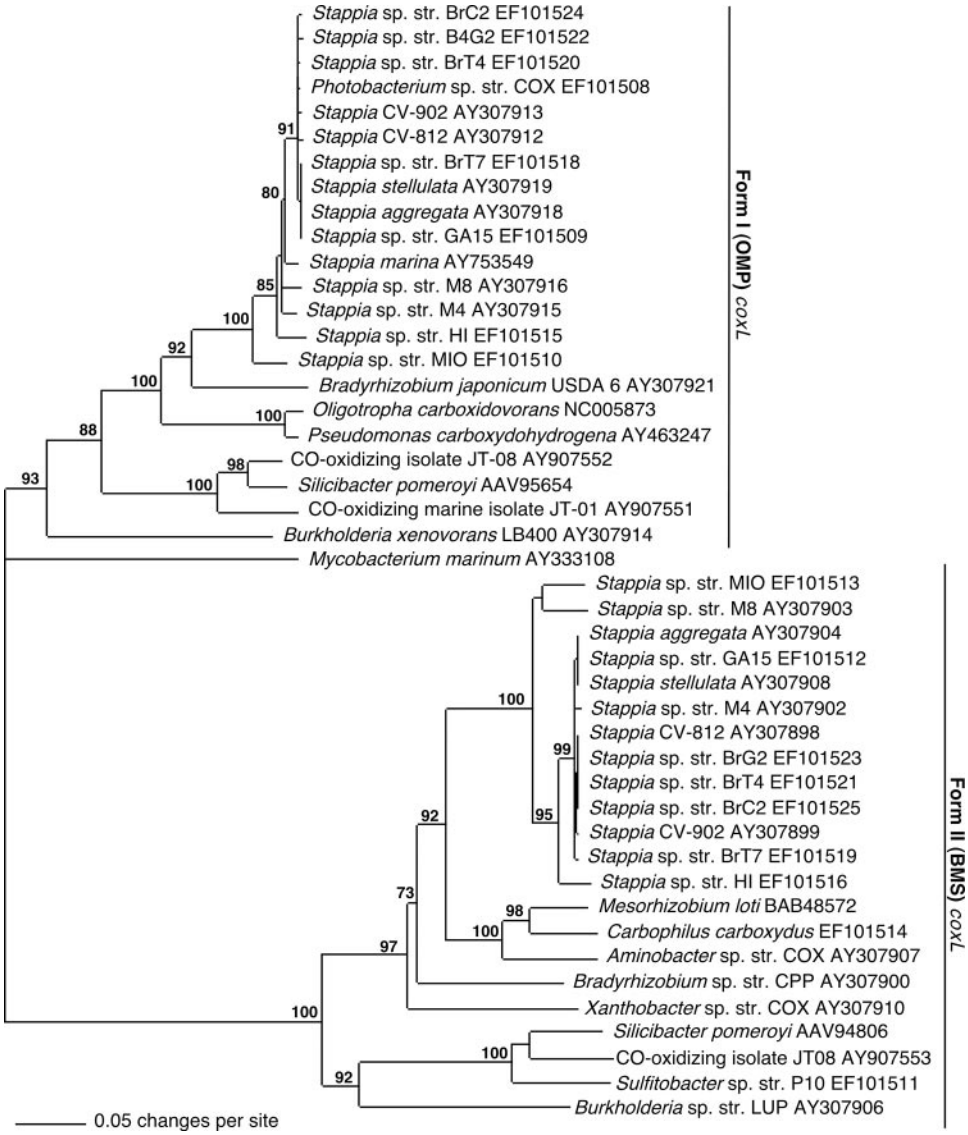

FIG. 5. Phylogram from neighbor-joining analysis (1,000 bootstrap replicates) of inferred OMP and BMS putative *coxL* amino acid sequences implemented with PAUP. *Mycobacterium marinum coxL* was used as an outgroup. Numbers at nodes indicate bootstrap support; values <70% are not shown.

date also contain form II putative *coxL* (Fig. 5). The form II protein appears to function as a CODH but may have a reduced capacity for CO oxidation and use an alternate substrate preferentially (15). CO utilization by *S. aggregata* conforms to a simple Michaelis-Menten kinetic model for low to moderate concentrations, but relatively high concentrations result in inhibition (Fig. 1), which may account for the inability of *Stappia* isolates to grow as typical carboxydotrophs.

In addition to using superambient concentrations (e.g., 1,000 ppm), strains GA15, HI, MIO, and M4 oxidize CO at concentrations significantly lower than those reported for seawater (Fig. 2) (37). Similar results have been obtained for other *Stappia* isolates (not shown). This suggests that CO may serve as a substrate for some *Stappia* strains under in situ conditions, perhaps supplementing the uptake of heterotrophic substrates, which typically occurs at only nanomolar to low micromolar concentrations.

In vitro CO consumption by *Stappia* strains and other CO oxidizers depends on incubation conditions (7, 8, 24, 28, 31). In *Pseudomonas carboxydoflava*, form I CODH is expressed constitutively and CO uptake occurs during batch heterotrophic growth on pyruvate (8). CODH is also expressed constitutively in *Mycobacterium* sp. strain JC1, and CO uptake occurs in the presence of several heterotrophic substrates, including glucose, but not pyruvate (28). Variability in expression and uptake also occurs for *Pseudomonas thermocarboxydovorans* (24), *Hydrogenophaga pseudoflava* (7), and *Oligotropha carboxidovorans*. Results presented here (Fig. 3) reveal the partial inhibition of activity by the addition of glucose to *Stappia aggregata* cells that are actively oxidizing CO. This effect appears due to allosteric

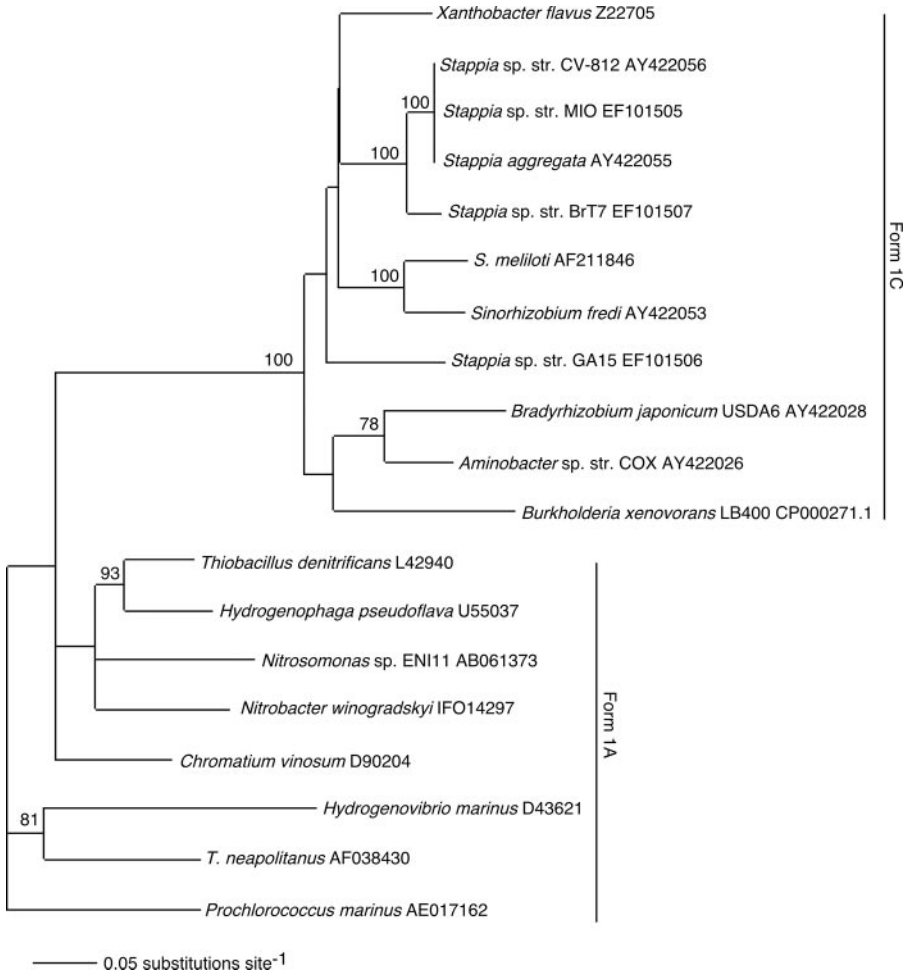

FIG. 6. Phylogram from neighbor-joining analysis (1,000 bootstrap replicates) of aligned nucleotide sequences implemented in PAUP for bacterial form I *cbbL* with bootstrap support indicated at nodes (values <70% not shown). *Prochlorococcus marinus* was used as an outgroup to root the tree for visual representation.

regulation by glucose or glucose metabolites, repression of CODH synthesis, or both and is consistent with results from *O. carboxidovorans* CODH expression studies (31).

Decreased CO oxidation due to the addition of 0.5 mM glucose (Fig. 3), which did not stimulate growth of *S. aggregata* (not shown), supports a role for allosteric inhibition. The repression of synthesis alone would leave cells with CODH levels (and presumably activities) similar to treatments without glucose. The repression of CODH synthesis by glucose concentrations of >0.5 mM is indicated by the fact that CO uptake per unit volume with 20 mM glucose is similar to that with 0.5 mM glucose, but cell density for the former increased by about 2.6-fold relative to the latter. An increase in cell density but not activity is consistent with maintenance of preexisting CODH without new synthesis. Regardless of the mechanism, inhibition is only partial, which suggests that under carbon-limited conditions in situ, CODH may be expressed and active, while diverse heterotrophic substrates are also used.

TABLE 4. S_{AB} derived from BOX genomic DNA fingerprinting analysis for *S. aggregata*, *S. stellulata*, and strains M4, M8, CV902, BrT4, GA15, MIO, and HI

Strain	Index of similarity for:							
	S. aggregata	M4	M8	CV902	BRT4	*S. stellulata*	GA15	MIO
M4	0							
M8	0	0.17						
CV902	0.11	0.13	0.15					
BrT4	0.13	0	0	0.15				
S. stellulata	0.12	0.29	0	0.13	0			
GA15	0.11	0	0.15	0.13	0.30	0		
MIO	0.10	0.12	0.13	0.22	0.13	0.35	0.22	
HI	0.11	0.4	0	0	0	0.13	0.13	0.11

The metabolic versatility of *Stappia* strains is illustrated by growth with a wide range of sugars, organic acids, aromatics, and amino acids, among others, and nitrate respiration or denitrification (Table 1) (1, 9, 14, 27, 29, 38). The potential for aromatic utilization reported here is consistent with prior analyses of *Stappia*-like isolates by Buchan et al. (3), who documented protocatechuic acid degradation. In addition, some *Stappia* isolates (those of *S. aggregata* and strains BrT7, CV812, GA15, MIO, and M4) possess *cbbL* (Fig. 6) and may be able to fix CO_2 via the Calvin cycle, possibly in conjunction with CO oxidation. The extent to which these two processes are coupled under in situ conditions remains to be determined.

Variability in substrate utilization and other phenotypic and biochemical traits (Tables 1 and 2), along with phylogenetic analyses, supports the designation of four new species. 16S rRNA gene analyses demonstrate that strains CV812, CV902, M8, BrC2, BrG2, BrT4, and BrT7 cannot be resolved from *S. aggregata* (Fig. 4) and contribute to a "species complex" that also includes isolates and uncultured bacterial clone sequences obtained from geographically and ecologically diverse sources. Strains GA15, HI, MIO, and M4 are distinct from this complex. Strain GA15 shares <97% 16S rRNA gene sequence similarity with *S. aggregata*, *S. alba*, *S. marina*, and *S. stellulata*, supporting its designation as a new species, *Stappia meyerae* sp. nov. It is united with *Stappia* through growth substrates, lipid profiles, polar flagellation, and enzymatic activities (Tables 1 and 2). Although its closest phylogenetic relatives include two *Roseibium* isolates, phylogenetic distance from them, growth substrates, gelatinase activity, and the absence of distinct pink pigmentation and peritricious flagellation differentiate *Stappia meyerae* sp. nov. from *Roseibium* (35).

Strain MIO 16S rRNA sequence is 96.2 and 97.6% similar to sequences from its closest phylogenetic neighbors, *S. aggregata* and *S. marina*, respectively. Phylogenetic distance (Fig. 4), lipid composition (Table 2), and the ability to denitrify and grow on terephthalate (Table 1) differentiate strain MIO from *S. aggregata*. Lipid composition and growth with glucose, mannose, mannitol, maltose, gluconate, and citrate differentiate strain MIO from *S. marina*. In addition, previous analyses involving *S. alba* (27) and *S. marina* (9) have shown that even 16S rRNA gene sequence similarities as high as 98.9% are associated with DNA-DNA hybridization values of ≪70% for congeneric *Stappia* species. Thus, these collective observations support the designation of a new species, *Stappia conradae* sp. nov.

Strain HI and M4 16S rRNA gene sequences share >97% similarity with the *S. aggregata* 16S rRNA gene sequence (97.6 and 97.7%, respectively), but both strains are clearly distinct from *S. aggregata* and all other validly described *Stappia* based on 16S rRNA and *coxL* phylogenies (Fig. 4 and 5), lipid profiles (Table 2), substrate utilization, and the presence of *cbbL* (and presumably the Calvin cycle) in *S. aggregata* but not strains HI or M4. The latter strains also denitrify, while *S. marina* does not. In addition, BOX-PCR results show substantial divergence between strains HI and MIO and all other *Stappia* isolates (Table 4). Collectively, these observations support two new species, *Stappia kahanamokuae* sp. nov. for strain HI and *Stappia carboxidovorans* sp. nov. for strain M4.

Growth substrate profiles not only provide valuable taxonomic information, but also offer important ecological insights. For instance, aromatic use, though variable among strains, is consistent with the isolation of *Stappia* spp. from phaeophyte macroalgae, which produce relatively high concentrations of polyphenols (25, 39). The recent identification of putative dehalogenase genes in the draft genome of *S. aggregata* (G. M. King et al., unpublished data) suggests a possible use of various alkyl or aryl halides, which are produced commonly by phytoplankton, macroalgae, and marine invertebrates (10, 23, 32, 36), in the growth of these organisms. The variable presence of *cbbL* genes indicates that some *Stappia* strains can function mixotrophically, supplementing organic carbon and energy sources with CO_2 fixation, presumably driven by CO oxidation. Other strains may simply use CO as a supplemental energy source.

In summary, the results presented here indicate that, in addition to its heterotrophic metabolism, the genus *Stappia* is characterized by its ability to oxidize CO at subambient to superambient levels, with or without mixotrophic CO_2 fixation. The ability of *Stappia* isolates to respire nitrate or denitrify, use numerous organics, and function in low to moderate salinities facilitates participation in both aerobic and anaerobic processes in carbon and nitrogen cycling in a wide range of marine environments. The results also support the establishment of four new species derived from geographically diverse habitats.

Description of *Stappia meyerae* sp. nov. *Stappia meyerae* (meyerae. L. fem. adj. *meyerae* of Meyer, honoring fundamental contributions by Ortwin Meyer, University of Bayreuth, to the physiology, biochemistry, and molecular biology of CO-oxidizing microbes).

Cells are aerobic, gram-negative, nonsporing, motile rods with single polar flagellums, 2.0 ± 0.03 μm in length and 0.8 ± 0.03 μm in width. Colonies circular, entire, slightly convex, smooth, cream to tan. Oxidase and catalase positive. Urease, β-galactosidase positive; esculin hydrolysis, gelatinase, indole production from tryptophan and arginine dihydrolase negative. Nitrate reduced to gas. Sodium required; optimum salinity for growth, 5 to 25 ppt. Principle fatty acids are 18:1ω7c and 11-methyl,18:1ω7c; G+C content, 57.8 ± 0.6%. Oxidizes carbon monoxide; contains large subunit gene for ribulose-1,5-bisphosphate carboxylase/oxygenase. Grows with acetate, alanine, aspartate, betaine, citrate, fumarate, fructose, galactose, glucose, gluconate, glucuronate, glutamate, glycerol, β-hydroxybutyrate, lactate, lactose, malate, malonate, maltose, mannitol, mannose, phthalate, propionate, pyruvate, proline, ribose, succinate, terephthalate, and valine. Does not grow with acetone, benzoate, 4-hydroxybenzoate, formate, glycine, isophthalate, isopropanol, methanol, tartrate, or mono-, di-, and trimethylamine. Weak growth on phenylalanine.

The type strain GA15 was isolated from *Ascophyllum nodosum* in the Damariscotta River (Maine).

Description of *Stappia conradae* sp. nov. *Stappia conradae* (conradae. L. fem. adj. *conradae* of Conrad, honoring important contributions by R. Conrad, Max Planck Institute for Terrestrial Biogeochemistry, Marburg, Germany, to the physiology and ecology of CO-oxidizing microbes in soil and aquatic environments).

Cells are aerobic, gram-negative, non-spore-forming, motile rods with single polar flagellums; 1.8 ± 0.1 μm in length and 0.7 ± 0.1 μm in width. Colonies circular, entire, slightly convex, smooth, cream to tan. Oxidase and catalase positive. Urease, β-galactosidase positive; esculin hydrolysis, gelatinase, indole production from tryptophan and arginine dihydrolase negative. Nitrate respired to nitrite; gas not produced. Sodium required; optimum salinity for growth, 15 to 35 ppt. Principle fatty acids are 18:1ω7c and 11-methyl,18:1ω7c; G+C content 58.2 ± 0.5%. Oxidizes carbon monoxide; contains large subunit gene for ribulose-1,5-bisphosphate carboxylase/oxygenase. Grows with acetate, alanine, citrate, fumarate, fructose, glucose, gluconate, glucuronate, glutamate, β-hydroxybutyrate, lactate, lactose, malate, maltose, mannitol, mannose, propionate, pyruvate, proline, succinate, and terephthalate. Does not grow with acetone, benzoate, 4-hydroxybenzoate, glycine, isophthalate,

isopropanol, methanol, phenylalanine, or mono- or dimethylamine. Weak growth on aspartate, betaine, formate, galactose, glycerol, malonate, phthalate, ribose, tartrate, and valine. Inhibited by trimethylamine.

The type strain MIO was isolated from a methanotrophic enrichment based on sediment obtained from a 160-m depth in Kagoshima Bay, Japan.

Description of *Stappia kahanamokuae* sp. nov. *Stappia kahanamokuae* (kahanamokuae. L. fem. adj. *kahanamokuae* of Kahanamokuae, honoring Hawaiian U.S. Olympic gold medalist and pioneering surfer, Duke Pahoe Kahinu Mokoe Hulikohola Kahanamoku).

Cells are aerobic gram-negative, nonsporing, motile rods with single polar flagellums; 2.1 ± 0.1 μm in length and 0.7 ± 0.1 μm in width. Colonies circular, entire, slightly convex, smooth, cream to tan. Oxidase and catalase positive. Urease, β-galactosidase, gelatinase positive; esculin hydrolysis, indole production from tryptophan and arginine dihydrolase negative. Nitrate reduced to gas. Sodium required; optimum salinity for growth, 35 ppt. Principle fatty acids are 18:1ω7c and 11-methyl,18:1ω7c; G+C content, 61.2 ± 0.1%. Oxidizes carbon monoxide; does not contain large subunit gene for ribulose-1,5-bisphosphate carboxylase/oxygenase. Grows with acetate, aspartate, betaine, citrate, fumarate, fructose, galactose, galacturonate, glucose, gluconate, glucuronate, glycerol, glycolate, β-hydroxybutyrate, lactate, lactose, malate, maltose, mannitol, mannose, propionate, pyruvate, proline, ribose, succinate, sucrose, terephthalate and valine. Does not grow with acetone, alanine, benzoate, ethanol, formate, 4-hydroxybenozate, glutamate, glycine, isophthalate, isopropanol, malonate, methanol, phenylalanine, phthalate, serine, or mono-, di-, and trimethylamine. Weak growth on tartrate.

The type strain HI was isolated from a surf water sample at South Point (Ka Lae), Hawaii.

Description of *Stappia carboxidovorans* sp. nov. *Stappia carboxidovorans* (car.box.i.do.vo.rans, L. n. *carbo*, charcoal, carbon; Gr. adj. *oxys*, sour, acid; L. v. *voro*, devour; M. L. part. adj. *carboxidovorans*, carbon acid devouring).

Cells are aerobic gram-negative, nonsporing, motile rods with single polar flagellums; 1.6 ± 0.1 μm in length and 0.8 ± 0.1 μm in width. Colonies circular, entire, slightly convex, smooth, cream to tan. Oxidase and catalase positive. Urease, β-galactosidase, gelatinase positive; esculin hydrolysis, indole production from tryptophan and arginine dihydrolase negative. Nitrate reduced to gas. Sodium required; optimum salinity for growth, 15 to 25 ppt. Principle fatty acids are 18:1ω7c and 11-methyl,18:1ω7c.; G+C content, 57.4 ± 0.3%. Oxidizes carbon monoxide; does not contain large subunit gene for ribulose-1,5-bisphosphate carboxylase/oxygenase. Grows with acetate, alanine, aspartate, betaine, citrate, fumarate, fructose, galactose, galacturonate, glucose, gluconate, glucuronate, glutamate, glycerol, glycolate, β-hydroxybutyrate, lactate, lactose, malate, malonate, maltose, mannitol, mannose, propionate, pyruvate, proline, ribose, succinate, sucrose, and valine. Does not grow with acetone, ethanol, formate, glycine, isophthalate, isopropanol, methanol, phenylalanine, phthalate, serine, tartrate, or mono-, di-, and trimethylamine. Inhibited by 4-hydroxybenzoate, serine, and terephthalate.

The type strain M4 was isolated from *Ascophyllum nodosum* from the Damariscotta River, Walpole, ME.

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