RESEARCH ARTICLE

REVISED *Stenotrophomonas goyi* sp. nov., a novel bacterium associated with the alga *Chlamydomonas reinhardtii* [version 3; peer review: 2 approved]

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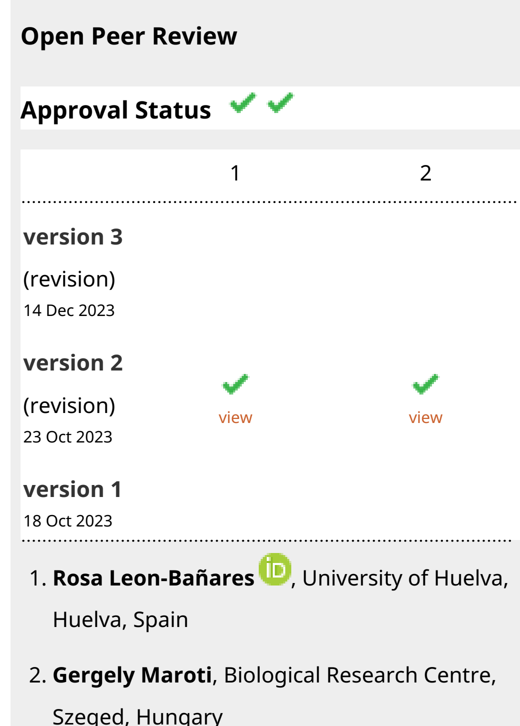

Abstract

Background

A culture of the green algae *Chlamydomonas reinhardtii* was accidentally contaminated with three different bacteria in our laboratory facilities. This contaminated alga culture showed increased algal biohydrogen production. These three bacteria were independently isolated.

Methods

The chromosomic DNA of one of the isolated bacteria was extracted and sequenced using PacBio technology. Tentative genome annotation (RAST server) and phylogenetic trees analysis (TYGS server) were conducted. Diverse growth tests were assayed for the bacterium and for the alga-bacterium consortium.

Results

Phylogenetic analysis indicates that the bacterium is a novel member of the *Stenotrophomonas* genus that has been termed in this work as *S. goyi* sp. nov. A fully sequenced genome (4,487,389 base pairs) and its

tentative annotation (4,147 genes) are provided. The genome information suggests that *S. goyi* sp. nov. is unable to use sulfate and nitrate as sulfur and nitrogen sources, respectively. Growth tests have confirmed the dependence on the sulfur-containing amino acids methionine and cysteine. *S. goyi* sp. nov. and *Chlamydomonas reinhardtii* can establish a mutualistic relationship when cocultured together.

Conclusions

S. goyi sp. nov. could be of interest for the design of biotechnological approaches based on the use of artificial microalgae-bacteria multispecies consortia that take advantage of the complementary metabolic capacities of their different microorganisms.

Keywords
algae, bacteria, consortia, cocultures, Chlamydomonas, Stenotrophomonas, vitamins, metabolic complementation

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REVISED **Amendments from Version 2**

A second accession number has been provided for the repository of *Stenotrophomonas goyi sp. nov.* The bacteria is now deposited in two internationally recognized culture collections.

The use of italics within the name of *Stenotrophomonas goyi sp. nov.* has been homogenized.

Two references have been updated.

Any further responses from the reviewers can be found at the end of the article

Introduction

The first described species of the *Stenotrophomonas* genus was *S. maltophilia*, which was a Gram-negative bacterium originally named as *Pseudomonas maltophilia*, and later transferred in 1993 to the new genus *Stenotrophomonas*, which was solely composed of *S. maltophilia.* In 2001, this species was moved to the genus *Xanthomonas* before it was finally moved back again in 2017 to its own genus when *Stenotrophomonas pictorum* was identified (Ryan *et al*., 2009; Wei *et al*., 2021). Currently, *Stenotrophomonas* is a genus comprising at least 19 validated species (https://lpsn.dsmz.de/genus/stenotrophomonas) (Parte *et al*., 2020). However, the molecular taxonomy of the genus is still somewhat unclear, and all its members are considered as "orphan species". All *Stenotrophomonas* spp. have shown intraspecific heterogeneity with high phenotypic, metabolic, and ecological diversity (Ryan *et al*., 2009).

The main reservoirs of *Stenotrophomonas* spp. are soil and plants, although they are ubiquitously present in different environments, including opportunistic human pathogens such as *S. maltophilia* (Ryan *et al*., 2009).

Stenotrophomonas spp. show promising potential for different biotechnological applications. Some *Stenotrophomonas* spp. are of interest to agriculture due to their ability to promote growth in different plant species. Some *Stenotrophomonas* spp. are even capable of establishing symbiotic relationships with plants. This plant growth promotion is related to the capacity of some *Stenotrophomonas* spp. to produce the plant growth hormone indole-3-acetic acid (IAA), fix nitrogen, oxidate elemental sulfur (S) to sulfate, or biocontrol plant pathogens (Banerjee and Yesmin, 2008; Park *et al*., 2005; Ryan *et al*., 2009; Suckstorff and Berg, 2003).

Moreover, they are also considered good candidates for bioremediation due to their tolerance to heavy metals and capability to metabolize a large variety of organic molecules, including phenolic and aromatic compounds (Liu *et al*., 2007; Mora-Salguero *et al*., 2019; Pages *et al*., 2008; Ryan *et al*., 2009). Finally, some *Stenotrophomonas* spp. can synthetize useful bioproducts such as antimicrobial and enzymes of biotechnological interest (Rivas-Garcia *et al*., 2022; Wolf *et al*., 2002).

Here we report the genome of *Stenotrophomonas goyi* sp. nov. isolated from a contaminated microalgae (*Chlamydomonas reinhardtii*) culture. This alga culture was simultaneously contaminated with *S. goyi, Microbacterium forte* (Fakhimi *et al*., 2023a) and *Bacillus cereus.* The metabolic interactions established between these four microorganisms are analyzed and discussed in a related publication where the ability of this multispecies consortium to sustain hydrogen production is highlighted (Fakhimi *et al*., 2023a).

Methods

Isolation of *Stenotrophomonas goyi* sp. nov.

This study took place at Campus Universitario de Rabanales, Cordoba, Spain. *S. goyi* sp. nov. where it was isolated from a fortuitously contaminated *Chlamydomonas reinhardtii* culture in the laboratory. Initially, the *Chlamydomonas reinhardtii* culture was simultaneously contaminated with three different bacteria (Fakhimi *et al*., 2023a). Individual members of this bacterial community were isolated by sequential rounds of plate streaking in Yeast Extract Mannitol (YEM) medium (handmade in our lab, described here), until three different types of bacterial colonies were visually identified. Colonies were grown separately, and the subsequent isolated DNA was used for PCR-amplification of their partial RNA 16S sequences. After sequencing, the three independently isolated bacteria were identified as members of the genus *Microbacterium*, *Stenotrophomonas*, and *Bacillus* (Fakhimi *et al*., 2023a).

Genome sequencing and assembling of *S. goyi* sp. nov.

DNA extraction and whole genome sequencing using PacBio (Pacific Biosciences) RS II Sequencing System (RRID: SCR_017988) were performed by SNPsaurus LLC (https://www.snpsaurus.com/). Whole genome sequencing generated 102,238 reads yielding 832,209,774 bases for 166 read depth over the genome (Table 1). Genome was assembled with Canu (RRID:SCR_015880) 1.7 (Koren *et al*., 2017) generating a 4,487,389 pb circular genome. The genome

Table 1. Main *de novo* sequencing and assembly statistics of *Stenotrophomonas goyi* sp. nov. genome.

Genome Size (pb)	Fold-coverage	GC content (%)	N° of contigs	Type	Plasmids
4,487,389	166	66.5	1	circular	No

completeness was checked by BUSCO 3.0.2 (RRID:SCR_015008) (Manni *et al*., 2021) and was 94.6% complete, with 94.6% of the genome single copy and 0.0% duplicated. Any other prokaryotic contamination was discarded using ContEst16S 1.0 (RRID:SCR_000595) (Lee *et al*., 2017).

Phylogenetic analysis

Phylogenetic analyses were performed using the Type (Strain) Genome Server (TYGS) at Leibniz Institute DSMZ (German Collection of Microorganisms and Cell Cultures GmbH) (Camacho *et al*., 2009; Farris, 1972; Kreft *et al*., 2017; Lagesen *et al*., 2007; Lefort *et al*., 2015; Meier-Kolthoff *et al*., 2022, 2013; Meier-Kolthoff and Göker, 2019; Ondov *et al*., 2016). Information on nomenclature, synonymy and associated taxonomic literature was provided by TYGS's sister database, the List of Prokaryotic names with Standing in Nomenclature (LPSN). Trees were inferred with FastME 2.1.6.1. Phylogenetic trees were drawn with iTOL (RRID:SCR_018174) (Letunic and Bork, 2021).

Annotation

Tentative annotation of the *S. goyi* sp. nov. genome was performed using the RAST tool kit, RASTtk (RRID: SCR_014606) at The Genome Annotation Service (Brettin *et al*., 2015; Overbeek *et al*., 2014). BlastKOALA service was used to automatically assign K numbers to the predicted proteins, which allowed Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology assignments, the putative characterization of individual gene functions, and the reconstruction of KEGG pathways (Kanehisa *et al*., 2016). The PHAge Search Tool Enhanced Release (PHASTER) (RRID: SCR_005184) was used to locate potential phage sequences within the *S. goyi* sp. nov. genome (Arndt *et al*., 2016)

Bacterium growth media and culturing conditions

Bacterial precultures were grown on Yeast Extract Mannitol (YEM) or Luria Broth (LB) media (handmade in our lab). Some growth experiments were done in Mineral Medium (MM) (Harris, 2008) supplemented with different nutrient sources (handmade in our lab). Tris-Acetate-Phosphate (TAP) medium (Harris, 2008) (handmade in our lab, described here) was also used occasionally. In some experiments, a vitamin cocktail (riboflavin, 0.5 $mg{\cdot}L^{-1}$; p-aminobenzoic acid, 0.1 $mg{\cdot}L^{-1}$; nicotinic acid 0.1 $mg{\cdot}L^{-1}$; pantothenic acid, 0.1 $mg{\cdot}L^{-1}$; pyridoxine, 0.1 $mg{\cdot}L^{-1}$; biotin, 0.001 $mg{\cdot}L^{-1}$; vitamin B12, 0.001 $mg{\cdot}L^{-1}$; thiamine, 0.001 $mg{\cdot}L^{-1}$) was added to bacterial cultures. More specific details for each experiment can be found in the corresponding figure and table legends. All the bacterium cultures were incubated at 24-28°C and under continuous agitation (130 rpm).

Coculturing algae and bacteria

Chlamydomonas cells were cultured for 3-4 days in TAP medium until mid-logarithmic growth phase, harvested by centrifugation (5.000 rpm for 5 min) and washed twice with fresh MM. Bacterial batch-cultures were incubated in TYM or LB medium until the Optical Density at 600 nm (OD_{600}) reached 0.8-1, then harvested by centrifugation (12.000 rpm for 5 min) and washed twice with fresh MM. Algae and bacteria were cocultured in 250 mL flasks containing 100 mL of the corresponding medium. Alga-bacterium cocultures were set to initial chlorophyll concentration of 10 $\mu g{\cdot}mL^{-1}$ for the alga and an initial OD_{600} of 0.1 for the bacterium. Algal and bacterial monocultures were used as controls. All cultures were incubated at 24°C with continuous agitation (120-140 rpm) and under continuous illumination (80 PPFD).

Determinations of algal and bacterial growth

The algal growth was assessed in terms of chlorophyll content. Chlorophyll measurements were done by mixing 200 µL of the cultures with 800 µL of ethanol 100%. The mix was incubated at room temperature for 2-3 min, and afterward centrifuged for 1 min at 12.000 rpm. The supernatant was used to measure chlorophyll (a + b) spectrophotometrically (DU 800, Beckman Coulter) at 665 and 649 nm (Wintermans and de Mots, 1965).

Bacterial growth in monocultures was estimated spectrophotometrically in terms of OD_{600} evolution (DU 800, Beckman Coulter). However, estimation of the bacterial growth in cocultures required bacterium cells separation from the alga cells. To do this, a customized Selective Centrifugal Sedimentation (SCS) approach was used. This approach consisted in finding the centrifugation parameters that led to maximize algal cell sedimentation while minimizing bacterial cell sedimentation (Torres *et al*., 2022). Thus, measuring the OD of the supernatant after centrifugation can provide an estimation of the bacterial growth in the cocultures. To do this, the percentages of precipitated cells of each monoculture were calculated at different forces (from 100 to 500 x g) and times (1 and 2 min) using the measured OD before (A_{BC}) and

after (A_{AC}) the centrifugation. Centrifugation at 200 x g for 1 min led to 87.9% of *Chlamydomonas* sedimentation, while only 2.1% of the bacterial cells dropped (meaning that 97.9% of the *S. goyi* cells remained in the supernatant). These parameters were chosen as a good compromise for SCS and used to evaluate the contribution of the bacteria to the OD in cocultures ($^{SCS}OD_{600}$).

Results

Identification of *Stenotrophomonas goyi* sp. nov.

A fortuitous contaminated *Chlamydomonas reinhardtii* culture (strain 704; CC-3597; https://www.chlamycollection.org/) was studied due to its enhanced hydrogen production capability. This alga culture turned out to be contaminated with three different bacterial strains (Fakhimi *et al.*, 2023), one of them consisting in a white-pigmented bacterium (Figure 1). This bacterium was isolated after several rounds of plate streaking in TYM medium. First, partial PCR amplification and sequencing of the ribosomal 16S gene allowed the identification of this bacterium as a member of the *Stenotrophomonas* genus. Afterwards, the whole genome sequence was obtained. Genome assembling provided one single circular contig of 4,487,389 pb (Table 1). No plasmids or extrachromosomal elements were identified.

The RAST server identified 4,147 genes (4,066 CDS + 81 rRNAs and tRNAs) (Table 2). Out of these 4,066 CDS identified by RAST, 1,096 of them were in subsystems. Tentative genome annotation derived from the RAST server is available in **Supplemental Table 1** as *Extended data* (González-Ballester *et al.*, 2023).

Phylogenetic analyses were performed with both, the whole genome (Figure 2) and the inferred 16S rDNAs (Figure 3). Pairwise comparisons with the closest type strains genomes are shown in Table 3. These phylogenetic analyses revealed

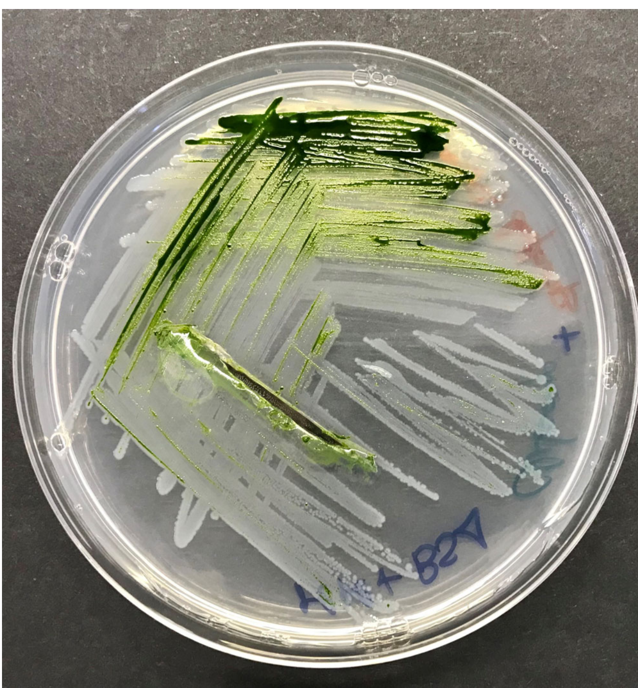

Figure 1. TAP plate with *Chlamydomonas reinhardtii* and *Stenotrophomonas goyi* sp. nov.

Table 2. Genome features of *Stenotrophomonas goyi* sp. nov. according to the RAST server. tRNA, transfer RNA; rRNA, ribosomal RNA.

Genes	CDS	tRNAs	rRNAs
4,147	4,066	71	10

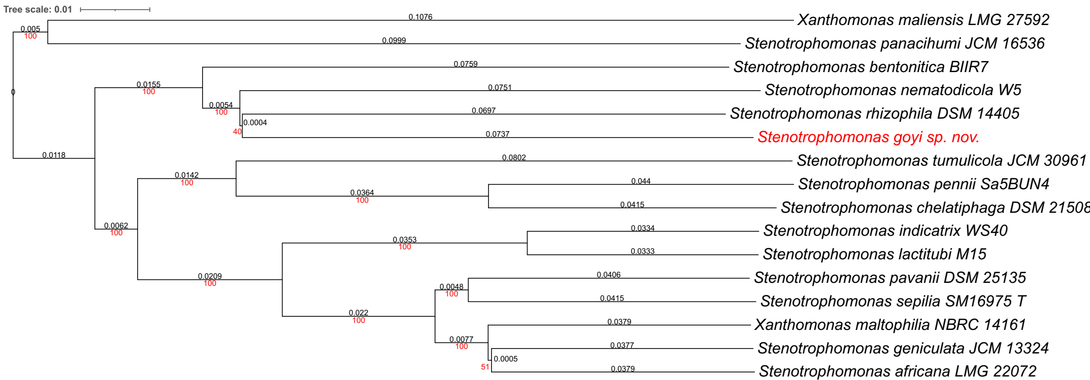

Figure 2. Phylogenetic tree for *Stenotrophomonas goyi* genome and related closest bacteria. Tree inferred with FastME 2.1.6.1 using the Genome BLAST Distance Phylogeny (GBDP) distances calculated from genome sequences. The branch lengths are scaled in terms of GBDP distance formula d5. The numbers above the branches are GBDP pseudo-bootstrap support values > 60% from 100 replications, with an average branch support of 91.6%. The tree was rooted at the midpoint. Branch lengths (black) and bootstraps (red) values are indicated. Genome sizes: 3,906,271–5,177,426 pb. Average δ statistics: 0.078 (Holland *et al*., 2002). Phylogenetic tree drawn with iTOL (Letunic and Bork, 2021).

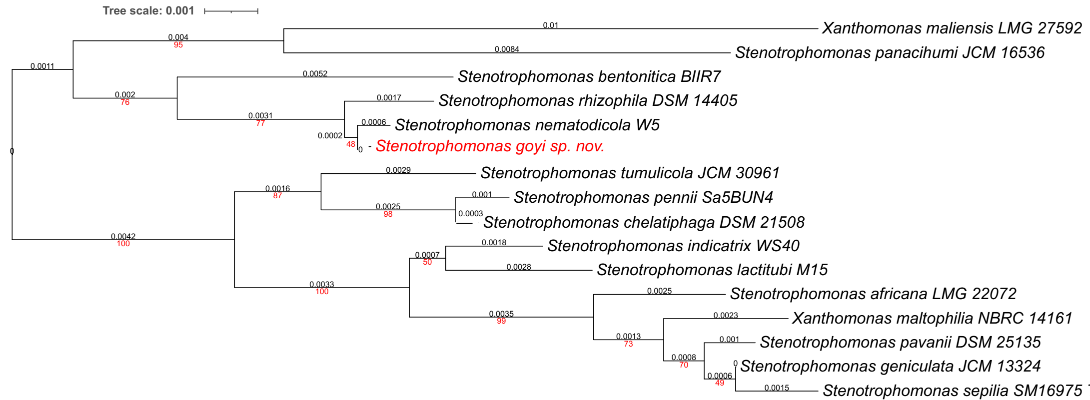

Figure 3. Phylogenetic tree for *Stenotrophomonas goyi* 16S rDNA and related closest bacteria. Tree inferred with FastME 2.1.6.1 using the Genome BLAST Distance Phylogeny (GBDP) distances calculated from 16S rDNA gene sequences. The branch lengths are scaled in terms of GBDP distance formula d5. The numbers above branches are GBDP pseudo-bootstrap support values > 60% from 100 replications, with an average branch support of 78.6%. The tree was rooted at the midpoint. Branch lengths (black) and bootstraps (red) values are indicated. RNA16S lengths: 1,385–1,535 pb. Average δ statistics: 0.236 (Holland *et al*., 2002). Phylogenetic tree drawn with iTOL (Letunic and Bork, 2021).

that the sequenced genome belonged to a new *Stenotrophomonas* sp.; all dDDH values (d0, d4 and d6) were below 70% (Meier-Kolthoff *et al*., 2013) (Table 3). This new bacterial species was named as *Stenotrophomonas goyi* sp. nov. The closest related bacteria in terms of whole genome and 16S rDNA similarities were *Stenotrophomonas rhizophila* DSM 14405 and *Stenotrophomonas nematodicola* W5, respectively (Figures 2 and 3). *S. goyi* sp. nov. genome was deposited in the NCBI as SUB12103906.

BlastKOALA (Kanehisa *et al*., 2016) service allowed KEGG orthology assignments to characterize individual gene functions and reconstruct KEGG pathways of *S. goyi* genome (**Supplemental Table 2;** *Extended data* (González-Ballester *et al.*, 2023)). Some important pathways were either absent or incomplete in *S. goyi* sp. nov. including assimilation of nitrate (the whole assimilatory pathway is missing including nitrate transporters) and sulfate (only sulfite reductase is present). On the other hand, putative complete pathways for the glyoxylate cycle and biosynthesis of biotin, coenzyme A, pantothenate, riboflavin, tetrahydrofolate, glutathione, pyridoxal-P, lipoic acid, dTDP-L-rhamnose, UDP-N-acetyl-D-glucosamine, C5 isoprenoids, bacterial lipopolysaccharides, and antimicrobial proteins, among others, were present. Incomplete pathways for the degradation of aromatic compounds (including phenol, toluene, xylene, methyl-naphthalene, 3-hydroxytoluene, and terephthalate) and myo-inositol biosynthesis, were also present.

Table 3. Pairwise dDDH values between *S. goyi* sp. nov. and the closest type strains genomes. The digital DNA-DNA Hybridization (dDDH) values are provided along with their confidence intervals (C.I.) for the three different Genome BLAST Distance Phylogeny (GBDP) formulas: a) formula d0: length of all High-Scoring segment Pairs (HSPs) divided by total genome length; b) formula d4: sum of all identities found in HSPs divided by overall HSP length; formula d6: sum of all identities found in HSPs divided by total genome length (Meier-Kolthoff *et al.*, 2013).

Closest strains to Stenotrophomonas goyi sp. nov.	dDDH (d0, in %)	C.I. (d0, in %)	dDDH (d4, in %)	C.I. (d4, in %)	dDDH (d6, in %)	C.I. (d6, in %)	G+C content difference (in %)
Stenotrophomonas rhizophila DSM 14405	50,6	[47.2-54.0]	30,9	[28.6-33.5]	45	[42.0-48.1]	0,78
Stenotrophomonas nematodicola W5	48,9	[45.5-52.4]	29,9	[27.5-32.4]	43,4	[40.4-46.4]	0,82
Stenotrophomonas bentonitica BIIR7	40,9	[37.5-44.3]	28,9	[26.5-31.4]	37,1	[34.1-40.1]	0,05
Xanthomonas maltophilia NBRC 14161	36,2	[32.8-39.7]	24,8	[22.5-27.3]	32,3	[29.3-35.4]	0,34
Stenotrophomonas sepilia SM16975 T	37,4	[34.0-40.9]	24,6	[22.3-27.1]	33,1	[30.1-36.2]	0,06
Stenotrophomonas lactitubi M15	37,7	[34.3-41.1]	24,6	[22.3-27.1]	33,3	[30.3-36.4]	0,63
Stenotrophomonas geniculata JCM 13324	36,4	[33.0-39.9]	24,4	[22.1-26.9]	32,3	[29.4-35.4]	0,34
Stenotrophomonas africana LMG 22072	35	[31.6-38.5]	24,4	[22.0-26.8]	31,3	[28.4-34.4]	0,21
Stenotrophomonas indicatrix WS40	39,2	[35.8-42.7]	24,4	[22.0-26.8]	34,2	[31.3-37.3]	0,09
Stenotrophomonas tumulicola JCM 30961	33,1	[29.7-36.7]	24,4	[22.0-26.8]	29,9	[27.0-33.0]	0,9
Stenotrophomonas pavanii DSM 25135	38,6	[35.3-42.1]	24,3	[22.0-26.8]	33,8	[30.9-36.9]	0,87
Stenotrophomonas chelatiphaga DSM 21508	36,6	[33.3-40.1]	24,2	[21.9-26.6]	32,4	[29.5-35.5]	0,32
Stenotrophomonas pennii Sa5BUN4	35,9	[32.5-39.4]	24	[21.7-26.5]	31,8	[28.9-34.9]	0,08
Stenotrophomonas panacihumi JCM 16536	26,7	[23.4-30.4]	22,4	[20.1-24.8]	24,7	[21.9-27.8]	2,37
Xanthomonas maliensis LMG 27592	19,3	[16.1-22.9]	21,5	[19.2-23.9]	18,8	[16.1-21.8]	0,32

Table 4. Growth of *S. goyi* sp. nov. on different nutrients. Mineral Medium (MM) was supplemented with different nutrients at 5 g·L^{-1} each, but methanol and ethanol (5 ml·L^{-1}). For acetic acid, Tris-Acetate-Phosphate (TAP) medium was employed (1.05 g·L^{-1} of acetic acid). Vitamins cocktail included riboflavin (0.5 mg·L^{-1}), p-aminobenzoic acid (0.1 mg·L^{-1}), nicotinic acid (0.1 mg·L^{-1}), pantothenic acid (0.1 mg·L^{-1}), pyridoxine (0.1 mg·L^{-1}), biotin (0.001 mg·L^{-1}), vitamin B12 (0.001 mg·L^{-1}), thiamine (0.001 mg·L^{-1}). ++, significant growth; +, poor growth; -, no growth.

Nutrients added	Growth
Sucrose	-
Lactose	-
Lactose + vitamins	-
Glucose	-
Glucose + vitamins	-
Mannitol	-
Lactic acid	-
Glycerol	-
Acetic acid (TAP medium)	-
Tryptone	++
Peptone	++
Yeast extract	++
BSA	++

Search with PHASTER (Arndt *et al.*, 2016) revealed one intact prophage (PHAGE_Erwini_phiEt88_NC_015295) located at position 753112-799783 of the *S. goyi* sp. nov. genome.

Nutrient requirements of *S. goyi* sp. nov.

S. goyi sp. nov. showed no growth on MM, or in MM supplemented with different C sources (sucrose, glucose, lactose, mannitol, or glycerol) (Table 4). The addition of vitamins to the MM supplemented with glucose or lactose did not support the bacterium growth either (Table 4). However, the bacterium showed an excellent growth when cultivated in MM supplemented with yeast extract, tryptone, peptone or even Bovine Serum Albumin (BSA) (Table 4), suggesting that this bacterium has a great capacity to use peptides/amino acids as C source, and probably also as N source. Moreover, the peptides/amino acids could also provide, in addition to C and N sources, other essential nutrients or even palliate potential amino acids auxotrophies. Note that MM medium has sulfate as only S source. As commented before, the genome of *S. goyi* sp. nov. is lacking a functional sulfate assimilation pathway. Thereby S-containing amino acids, such as cysteine and methionine, could support the growth in medium rich in peptides/amino acids.

To confirm this hypothesis, *S. goyi* sp. nov. was inoculated in plates of MM + glucose supplemented with different combinations of cysteine, methionine, biotin, and thiamine. Only plates containing cysteine and methionine supported the bacterial growth for several culturing rounds (Figure 4). This result confirms the cysteine and methionine growth dependence of *S. goyi.* sp. nov. Cysteine and methionine could provide either a reduced S source or complement an auxotrophy for these two amino acids. Since *S. goyi* sp. nov. genome has complete pathways for all the essential amino acids, is more likely that cysteine and methionine are being used as reduced S sources. Similar results were found for *M. forte*, where cysteine and methionine are required as S sources (Fakhimi *et al.*, 2023a).

S. goyi sp. nov. showed optimal growth between 24 and 32°C and pH 5-9 (Table 5). Despite the presence of the complete multidrug resistance efflux pump MexJK-OprM in the genome (Chuanchuen *et al.*, 2005), no resistance to tetracycline, rifampicin, chloramphenicol and polymyxin (50 µg/mL each) was observed.

Growth of *S. goyi* sp. nov -*C. reinhardtii* consortium

Torres *et al.* (2022) reported that cocultures of *S. goyi* sp. nov. (published as *Stenotrophomonas* sp.) and *C. reinhardtii* promoted the growth of the microalga (nearly doubled) when incubated in MM supplemented with glucose and mannitol, but not when supplemented with acetic acid.

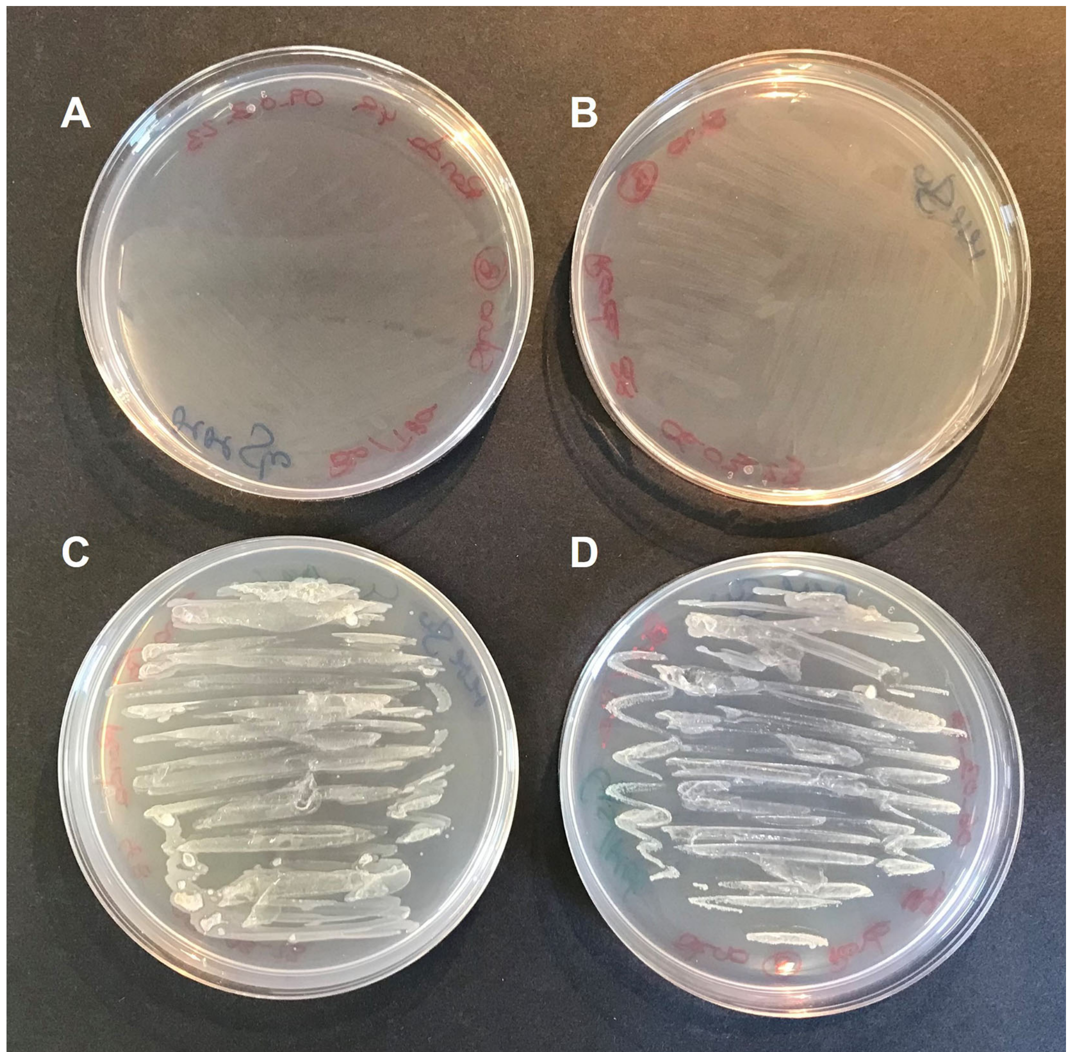

Figure 4. Cysteine and methionine requirements of *S. goyi* to grow. *S. goyi* sp. nov. was inoculated in: A) plates of Mineral Medium (MM) + glucose (5 g·L^{-1}); B) MM + glucose + biotin (0.001 mg·L^{-1}) + thiamine (0.001 mg·L^{-1}); C) MM + glucose + cysteine (4 mM) + methionine (4 mM); and D) MM + glucose + cysteine + methionine + biotin + thiamine.

Table 5. Growth of *S. goyi* sp. nov. at different temperatures and pHs. Lysogeny broth (LB) medium was used in all the conditions.

Temperature °C	Growth	pH	Growth
10	-	3	-
15	+	4	+
20	++	5	+++
24	+++	6	+++
28	+++	7	+++
32	+++	8	+++
37	++	9	+++
42	-	10	+
		11	-

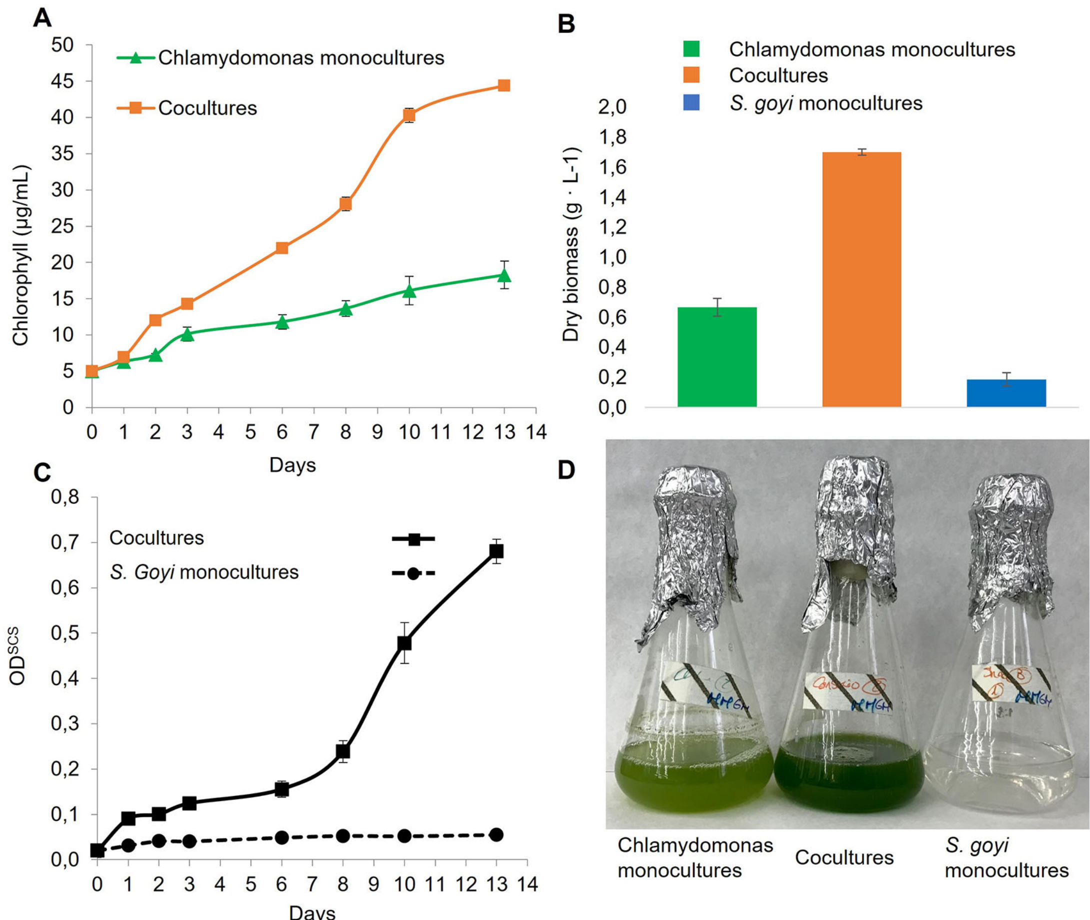

Figure 5. ***S. goyi*** **sp. nov. and** ***C. reinhardtii*** **growth in consortium.** *S. goyi-C. reinhardtii* consortium, and respective control monocultures, were incubated in Mineral Medium (MM) supplemented with glucose (5 g·L^{-1}) and mannitol (5 g·L^{-1}). A) Chlorophyll content; B) dry weight biomass after 13 days; C) bacterial growth in terms of OD^{SCS}; D) actual picture of the cultures after 13 days.

Here, it was also checked if the bacterium also benefited when co-cultivated with *C. reinhardtii* in glucose- and mannitol-containing media. First, we observed that the chlorophyll content in the cocultures was 2.4 times higher than in the *C. reinhardtii* monocultures after 13 days (Figure 5A), which is in accordance with previous results (Torres *et al*., 2022). Additionally, the dry biomass resulting from the consortia was 2.2 times higher than the sum of the respective monocultures (Figure 5B). Finally, the growth of the bacterium in cocultures was very efficient, unlike *S. goyi* sp. nov. monocultures (Figure 5C).

These results indicate that *S. goyi* sp. nov. and *C. reinhardtii* can establish a mutualistic relationship when incubated in sugars-containing media. On the one hand, *S. goyi* sp. nov. can greatly support the growth of the *C. reinhardtii* in media supplemented with glucose or mannitol, which are two carbon sources that the alga cannot utilize. Likely, this growth promotion is due to the release of acetate and/or CO_2 from the bacteria after the sugar fermentation. Acetate is the sole organic carbon source that *C. reinhardtii* can utilize during heterotrophic/mixotrophic growth (Chaiboonchoe *et al*., 2014). On the other hand, *S. goyi*, sp. nov. can grow in media without amino acids/peptides supplementation when cocultured with *C. reinhardtii*, suggesting that the alga must provide some essential nutrients for the bacterium. Reduced S forms excreted by the alga (*e.g.*, cysteine or methionine) could potentially explain the bacterium growth in the consortium.

Discussion and conclusions

Stenotrophomonas spp. are common constituents of the rhizosphere, and their potential for agricultural biotechnology is arising. However, their association with algae is poorly explored. Most plant growth-promoting bacteria (PGPB) are firstly identified in the rhizosphere and in association with plants. However, many PGPB are then also often commonly

found in association with algae. This is likely reflecting that the kind of relationships established between bacteria and plants are similar to the relationships between bacteria and algae. This could potentially be the case for *Stenotrophomonas* spp., although the relative poor taxonomic curation and heterogeneity of the genus may prevent the tracking of its association with algae.

Some *Stenotrophomonas* spp. show a limited nutritional range, while others are capable of metabolic versatility (Ryan *et al*., 2009). *S. goyi* sp. nov. is unable to grow in the absence of a source of peptides/amino acids, which imply that in natural ecosystems it may rely on other microorganisms to obtain essential nutrients. As stated before, *S. goyi* sp. nov. is unable to use sulfate as S source. The peptides/amino acids are likely providing S-reduced forms (such as cysteine and methionine) to *S. goyi* sp. nov.

Stenotrophomonas goyi sp. nov. was isolated from an alga culture (*C. reinhardtii*) that showed an enhanced capacity to produce hydrogen and biomass when incubated in mannitol and yeast extract containing medium (Fakhimi *et al*., 2023a). This algal culture was simultaneously contaminated with two other bacteria: *Microbacterium forte* and *Bacillus cereus.* Out of the three bacteria, *M. forte* was the main responsible for the enhanced algal hydrogen production. However, *C. reinhardtii-M. forte* cocultures were unable to produce hydrogen and biomass concomitantly. In addition to *M. forte*, the presence of *S. goyi* sp. nov. and *B. cereus* in the cocultures was needed to produce jointly hydrogen and algal biomass (Fakhimi *et al*., 2023a), which stresses the biotechnological interest of *S. goyi* sp. nov.

M. forte showed auxotrophy for biotin and thiamine, and like *S. goyi* sp. nov. was unable to grow on inorganic S sources (Fakhimi *et al*., 2023a). In this multispecies association, *S. goyi* sp. nov. and *C. reinhardtii* could alleviate the auxotrophy of *M. forte* sp. nov. for biotin and thiamine. *S. goyi* sp. nov. could also provide ammonium derived from the mineralization of the amino acids to the alga. On the other hand, the alga could provide S-reduced sources such as cysteine and methionine for *S. goyi* sp. nov. and *M. forte.* In any case, this multispecies association was mutually beneficial and prevented an excessive bacterial growth in cocultures, which could be one of the main drawbacks when algae-bacteria cocultures are used for biotechnological applications.

Nevertheless, the precise metabolic relationships established in this multispecies consortium that led to the extension of the *C. reinhardtii* cells viability during hydrogen production condition is not yet unraveled and need to be further investigated.

Ethical considerations
Not applicable.

Data availability
Underlying data
Spanish Type Culture Collection (CECT): *Stenotrophomonas goyi* sp. nov. bacterium. Accession number CECT30764; https://www.cect.org/vstrn.php?lan=en&cect=30764 (Universidad de Cordoba, 2022).

NCBI Genome: *Stenotrophomonas goyi* sp. nov. genome sequence. Accession number CP124620; https://identifiers.org/ncbi/insdc:CP124620 (Fakhimi *et al.*, 2023b).

Extended data
Zenodo: Supplemental Files, https://doi.org/10.5281/zenodo.8091305 (González-Ballester *et al.*, 2023).

This project contains the following extended data:

- Supplemental Table 1 [Tentative annotation of the *S. goyi* sp. nov. genome]

- Supplemental Table 2 [KEGG orthology assignments]

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

Acknowledgments
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Gergely Maroti
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The manuscript describes the characteristics of a novel *Stenotrophomonas* sp. bacterium isolated from a complex algal-bacterial association. The genome description is clear and concise, the nutrient requirements of the bacterium were also clearly determined.

The mutualistic growth promoting effects were proven experimentally. The potential molecular mechanisms behind the observed effects are not shown or hypothesized, however, it is not the goal of this manuscript.

The conclusions are correct, the application of multispecies consortia including bacteria and green algae is a promising way for biological hydrogen production in an economically viably manner.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Algal biotechnology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 27 November 2023

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The paper is clearly presented and describes an interesting study, which is detailed presented. In this paper, the authors describe the characterization of a new bacterial species of the genus Stenotrophomonas, found as contaminant in a culture of the chlorophyte Chlamydomonas reinhardtii. The bacterial genome has been sequenced and tentatively annotated. Phylogenetic trees based on both whole genome and 16S rDNA sequences were stablished. The growth of the new bacteria, either with or without the microalga, was evaluated.

Functional annotation of the genome of the new bacteria reveled that some important pathways are not present in S. goyi genome. This data, beside several growth test carried out with aminoacids and /or peptide hydrolysates make the authors suggest that peptides and amino acids are a good C source, and probably also a good N or S source, for S. goyi, which can not grow in minimal medium not supplemented with aminoacids or peptides. Addition of aminoacid/peptides or co-cultivation with the microalga is necessary for the growth of this bacterial. Moreover, the microalga growth rate is practically doubled when cultured in the presence of the bacteria. The authors propose the establishment of a mutualistic relationship between C. reinhardtii and the bacteria, in which the bacteria would provide a carbon source (acetate or CO2 obtained from bacterial sugar fermentation) to the microalgae and the bacteria would obtain S-aminoacids excreted by the alga.

The authors suggest that many microalgal-associated bacteria can promote higher plant growth, being the characterization of this algal-bacterial consortium of interest to identify potent plant growth-promoting bacteria.

Suggestions:

- The assessment of the bacterial growth by determination of optical density is adequate for pure cultures, but it is difficult to carry out in mixed algal-bacterial cultures. The authors have optimized and validated a selective centrifugal sedimentation approach to separate bacteria and microalgae. Although the approach seems to work well, I would suggest determination of colony forming units (CFU) to follow bacterial growth in future occasions. In may experience, the sedimentation properties of the microalga can change along the growth cycle

- Indicate in the legend of Figure 1, that shows a plate with Chlamydomonas reinhardtii and Stenotrophomonas goyi sp. nov., the culture medium used. Is it bacterial or algal growth médium? Is it MM or has been supplemented with sugars?

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Biochemistry and Biotechnology of microalgae

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Comments on this article

Version 2

Reader Comment 22 Nov 2023

Tiago Henriques, University of Coimbra, Coimbra, Portugal

In the "Data availability" section, "NCBI Gene" has the accession number **CP116871** which directs to "Microbacterium sp. A(2022) strain A chromosome, complete genome". I think the accession nummber that should be there is **CP124620**.

Competing Interests: No competing interests were disclosed.

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