The freshwater microalga Chlamydomonas reinhardtii, which lives in wet soil, has served for decades as a model for numerous biological processes, and many tools have been introduced for this organism. Here, we have established a stable nuclear transformation for its marine counterpart, Chlamydomonas sp. SAG25.89, by fusing specific cis-acting elements from its Actin gene with the gene providing hygromycin resistance and using an elaborated electroporation protocol. Like C. reinhardtii, Chlamydomonas sp. has a high GC content, allowing reporter genes and selection markers to be applicable in both organisms. Chlamydomonas sp. grows purely photoautotrophically and requires ammonia as a nitrogen source because its nuclear genome lacks some of the genes required for nitrogen metabolism. Interestingly, it can grow well under both low and very high salinities (up to 50 g · L⁻¹) rendering it as a model for osmotolerance. We further show that Chlamydomonas sp. grows well from 15 to 28°C, but halts its growth at 32°C. The genome of Chlamydomonas sp. contains some gene homologs the expression of which is regulated according to the ambient temperatures and/or confer thermal acclimation in C. reinhardtii. Thus, knowledge of temperature acclimation can now be compared to the marine species. Furthermore, Chlamydomonas sp. can serve as a model for studying marine microbial interactions and for comparing mechanisms in freshwater and marine environments. Chlamydomonas sp. was previously shown to be immobilized rapidly by a cyclic lipopeptide secreted from the antagonistic bacterium Pseudomonas protegens PF-5, which deflagellates C. reinhardtii.

Key index words: electroporation; marine Chlamydomonas; microalgae; microbial interactions; nitrogen metabolism; nuclear transformation; salinity; temperature

Abbreviations: Actin, Act; Amt, Ammonium transporter; Aph7", Aminoglycoside phosphotransferase; Gln, Glutamine synthetase; InDel, Insertion/Deletion; KH, K homology; Nia, Nitrate reductase; Nii, Nitrite reductase; RRM, RNA recognition motif; RT-
Eukaryotic photosynthetic unicellular organisms, known as microalgae, contribute significantly to carbon fixation on Earth and form the basis of food webs in freshwater and marine ecosystems (Field et al. 1998). They can be found in aquatic environments, wet soil, or soil crusts, and they are exposed to ever-changing abiotic conditions such as temperature and light. In nature, they co-exist with other microorganisms, such as bacteria and fungi, which can have an impact on their survival and growth rates. To study and understand the influence of abiotic and biotic factors within the hundreds of thousands of different microalgae in detail, we need several microalgal model systems with established molecular tools that can be manipulated genetically. For decades, the unicellular green biflagellated alga *Chlamydomonas reinhardtii*, which lives in wet soil (reviewed in Sasso et al. 2018), has been in constant use and developed as a model to study, among others, photosynthesis, the structure and function of the flagella, as well as light- and temperature-driven processes (reviewed in Salomé and Merchant 2019). Recently, *C. reinhardtii* has also been used to study biotic interactions with antagonistic heterotrophic bacteria. It has been shown that specific secondary metabolites produced by *Pseudomonas protegens* attack the algal cells and inhibit their mobility and growth (Aiyar et al. 2017). One of these, the cyclic lipopeptide Orfamide A, causes an increase in cytosolic Ca$^{2+}$, which leads to the deflagellation and subsequent immobilization of *C. reinhardtii* within minutes (Aiyar et al. 2017). Other known secondary metabolites of *P. protegens* PF-5 present in a bacterial extract are involved in its growth arrest and it has yet to be determined which are responsible for this step. Interestingly, the identified lipopeptide was also active in an aquatic marine *Chlamydomonas* species (*Chlamydomonas* sp. SAG25.89), which was immobilized rapidly as well upon treatment. *Chlamydomonas* sp. SAG25.89, verified to be *Chlamydomonas* sp. CCMP235 (Fig. S1a in the Supporting Information), was isolated from the Nantucket Sound (USA; https://ncma.bigelow.org/ccmp235). This marine biflagellated green alga offers the possibility to compare the interactions and mechanisms studied in the freshwater *Chlamydomonas* species in the marine environment.

Marine green *Chlamydomonas* species have been the focus of research on other important biological questions recently. The Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP) aimed to shed light on the functional diversity of eukaryotic life in the oceans (Keeling et al. 2014). Thereby, *Chlamydomonas euryale*, a marine species discovered by Lewin in 1957 (Lewin 1957) in Nova Scotia, Canada, and also isolated in the Yellow Sea (https://ncma.bigelow.org/ccmp219) was one of two *Chlamydomonas* species to be selected in this project (BioSample: SAMN02739957; BioProject ID: PRJNA231566). This species also served as a marine green alga model for studies on fatty acid and polyketide synthesis (Kohli et al. 2016). Another marine strain, *Chlamydomonas* sp. UWO241, which is found in the arctic area, tolerates low temperatures, hypersalinity, and extreme shade, and revealed special trades such as an altered organization of the photosystem I (Kalra et al. 2020). In addition, *Chlamydomonas* sp. KNM0029C, an additional arctic strain, has been used for the co-production of biodiesel and bioethanol (Kim et al. 2020). Some *Chlamydomonas* species from coastal subtropical areas have also been recently sequenced, which were found to have especially abundant genes involving sulfate transport, sulfotransferase, and glutathione S-transferase activities in their genomes (Nelson et al. 2019).

To study the biotic and abiotic interactions of algae in their environment, investigate their evolution, and efficiently perform algal-based biotechnology processes, we need a variety of algal models. To date, only a few algal model organisms have emerged, in which not only their genome and/or transcriptome sequence data are available, but also whose genome can be transformed (Grossman et al. 2007, Cock and Coelho 2011, Chang et al. 2016, Salomé and Merchant 2019, Falcioni et al. 2020). For marine green algae, this includes the non-flagellated marine picoeukaryote *Ostreococcus tauri* (Keeling 2007, van Ooijen et al. 2012, Lozano et al. 2014, Sanchez et al. 2019), as well as the extreme halotolerant cell wall-less green alga *Dunalieilla salina*, which lives in sea salt fields (Song et al. 2019); and the green macroalga *Ulva mutabilis*, which relies on biotic interactions with bacteria to shape its structure (Oertel et al. 2015, Wichard et al. 2015). Very recently, a transformation system has also been established in some archiplastidal marine species, including *Micromonas* and *Tetraselmis* (Faktorová et al. 2020).

Here, we aimed to set up a model for a marine *Chlamydomonas* as a typical cell wall containing, biflagellated unicellular alga using *Chlamydomonas* sp. SAG25.89. With this model, we will be able to study its biotic and abiotic interactions at a molecular level and compare them with the freshwater counterpart *C. reinhardtii*. We first determined the growth conditions for *Chlamydomonas* sp. SAG25.89 and found that it tolerates a broad range of salinities, requires ammonium as a nitrogen source, grows purely photoautotrophically, and halts its growth at 32°C. We further defined hygromycin and paromomycin as usable targets for selection markers and obtained the first sequencing data of its nuclear genome. We cloned the relevant cis-acting elements of its *Actin* (*Act1*) gene and used them to design
and assemble a transformation vector with a Aph7" resistance cassette. Finally, we established a transformation protocol involving a high-quality electroporation system with several variables used for stable nuclear transformation of Chlamydomonas sp. SAG25.89, taking advantage of its ability to survive at low and high salinity levels.

MATERIALS AND METHODS

Strains and culture conditions. Chlamydomonas reinhardtii SAG73.72 and Chlamydomonas sp. SAG25.89 were obtained from the “Sammlung von Algenkulturen” (SAG) of the University of Göttingen. Chlamydomonas euryale CCMP219 and Chlamydomonas sp. CCMP255 were obtained from the National Center for Marine Algae and Microbiota (NCMA). Culturing of C. reinhardtii was performed as described previously (Li et al. 2018). Cultures of Chlamydomonas sp. and C. euryale were grown as indicated, either under LD16:08 or under LD12:12 with a light intensity of 60 μmol · m⁻² · s⁻¹ at 18°C unless otherwise indicated. The medium used was a modification of the 3N-BBM + V medium (Bischoff and Bold 1963), in which the nitrate has been replaced in some cases by 17 mM NaNO₃ or 17 mM NH₄Cl and 20 mM of HEPES, pH 8, as indicated. We refer to the latter medium that was used routinely as NH₄-BBM. In the cases when extra salt was added, NH₄-BBM is followed by the concentration of salt added (e.g., NH₄-BBM + 25 g · L⁻¹ NaCl).

Genomic DNA extraction and library preparation for Illumina sequencing. The genomic DNA extraction was performed using NucleoSpin Plant II (MACHEREY-NAGEL) kit with an initial biomass of 2 g algal cells obtained from a 10-d-old culture of each strain (Chlamydomonas sp. SAG25.89 and C. euryale, respectively). DNA integrity and fragment lengths were verified using an Agilent 2100 Bioanalyzer system. Illumina libraries were prepared using the TrueSeq DNA Nano kit according to the manufacturer’s instructions. The resulting two libraries were multiplexed and sequenced in rapid mode on one lane of an Illumina HiSeq2500 platform, yielding 100 bp paired-end reads.

Genomic DNA extraction and library preparation for PacBio sequencing. The genomic DNA extraction was performed using a QIAGEN Genomic-tip 20/ G kit with an initial biomass of 2 g obtained algal cells from a 10-d-old culture of each strain. DNA integrity and fragment lengths were checked using an Agilent 2100 Bioanalyzer system. Prior to sequencing, short fragments were removed using the BluePippin system. Then, long-read library preparation and sequencing were performed following the manufacturer’s instructions for a PacBio RS II platform using four SMRTCells per strain.

Preliminary genome draft of Chlamydomonas euryale and Chlamydomonas sp. SAG25.89. For each strain, we generated a preliminary draft assembly using long-read data. The raw reads from four PacBio SMRTCells per strain were combined, quality controlled with FastQC v0.11.7, and finally assembled using Canu 1.5 (Koren et al. 2017) with the following parameters: correctedErrorRate = 0.06 genotypeSize = 120M. No further polishing or error-correction was performed, and the Illumina data were not used in the assembly process. We assessed the quality of these two assembly drafts with QUAST v4.4 (Gurevich et al. 2013).

Phylogenetic analysis of the 18S rRNA genes of Chlamydomonas and related species. For the phylogenetic analysis of the 18S rRNA gene, we used the sequences available for various known Chlamydomonas and related species with the names given after the several reclassifications that the Chlamydomonas genera have undergone (Lewin 1957, Nakazawa et al. 2001, Pröschold et al. 2001, 2005, Buchheim et al. 2003, 2013, Pocock et al. 2004, Eddie et al. 2008, Demchenko et al. 2012, Yumoto et al. 2013, Nakada and Tomita 2014, Lemieux et al. 2015, Mertens et al. 2015, Wang et al. 2016, Munakata et al. 2016, Nakada et al. 2016, Watanabe and Lewis 2017; Appendix S1 in the Supporting Information). A Multiple Sequence Alignment (MSA) was calculated using MAFFT (Katoh and Standley 2013) using the E-INS-I algorithm and enabled sequence direction correction. Otherwise, the default values were used. The 5' and 3' ends of the sequences were trimmed, and only the region available for all species was kept.

Evolutionary analyses were performed with MEGA X (Kumar et al. 2018). The phylogenetic tree with the highest log likelihood was derived using the Maximum Likelihood method and the General Time Reversible model (Nei and Kumar 2000). The tree with the highest log likelihood was calculated. The percentage of trees, in which the associated taxa clustered together, is shown next to the branches (bootstrap value of 1000). The initial tree(s) for the heuristic search were determined automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with the superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (16 categories). The rate variation model allowed for some sites to be evolutionarily invariable (1 + I).

Comparison of the 18S and 26S rRNA genes. We used the preliminary draft of an assembled genome of Chlamydomonas sp. SAG25.89 and C. euryale to pinpoint and extract the rDNA loci. The PacBio reads were mapped back to the fragment using the default values of minimap2 (Li 2018) using the -map-pb option. The Illumina reads were mapped using the default values of BWA-mem (Li 2013). The mapped reads were then extracted and SPAdes 3.12 (Antipov et al. 2016) was used for hybrid assembly, selecting the options --only-assemble, --careful, and multi-k-nmer sizes of 81,85,89,93, and 97. The resulting contig was used as the final rDNA.

Comparisons between Chlamydomonas sp. and C. euryale were achieved by pairwise alignment between the contigs using MAFFT (Katoh and Standley 2013) with the G-INS-I algorithm. Comparisons of the 18S and 26S rDNA from Chlamydomonas sp. CCMP255 were performed with the same procedure but using the sequences found in the NCBI database (Accession no. DQ009754 and DQ015721).

Analysis of the poly(A) signal. The transcriptome of Chlamydomonas euryale from the iMicrobiome project (Accession number: MMETSP00063) was used together with an in-house script (Appendix S2 in the Supporting Information). The analysis was performed in three steps. In the first step, the mRNAs containing a poly(A) tail with a length equal or longer than one A were included and then clustered according to the default values of minimap2 (Li 2018) using the -map-pb option. The Illumina reads were mapped using the default values of BWA-mem (Li 2013). The mapped reads were then extracted and SPAdes 3.12 (Antipov et al. 2016) was used for hybrid assembly, selecting the options --only-assemble, --careful, and multi-k-nmer sizes of 81,85,89,93, and 97. The resulting contig was used as the final rDNA.

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to see whether there was any positional enrichment of the motifs in the sequence.

Evaluation of the different genes from Chlamydomonas euryale. To determine gene positions in the draft genome of Chlamydomonas euryale, we used tBLASTn with the default parameters. The C. reinhardtii protein sequences were used as query and the genome draft of C. euryale as the target. It is generally accepted that an identity of 30–40% in long protein alignments (100 aa or longer) is significant to consider two proteins as homologs, while 20–35% is considered the twilight zone and structural information is needed to infer homology (Kost 1999, Pearson 2015). We set the cutoff value at 40% identity as the strains are reported to be related. The second criterion used was the minimum query coverage. We binned the results in four groups, higher or equal to 75%, between 50 and 75%, between 20 and 50%, and lower than 20%. For all the candidates, a fragment including 1,000 bp upstream and downstream from the hit region was used. The DNAseq data were mapped to the fragment using BWA-mem (Illumina reads) and Minimap2 (PacBio). The mapped reads were then filtered and quality-trimmed using Trimomatic (Bolger et al. 2014) with the parameters LEADING:24; TRAILING:24; SLIDINGWINDOW:4,28; MINLEN:81. The resulting reads were used to reassemble the fragment using SPAdes 3.12 (Antipov et al. 2016) with the—only assemble and—careful options enabled and the k-mer sizes of 81, 85, 89, 93, and 97. Afterward, the RNAseq data were mapped using HISAT2 v2.1 (Kim et al. 2019) to the curated genomic fragment, and the intron/exon borders were established.

Evaluation of the different genes from Chlamydomonas sp. SAG25.89. For the curation of the genomic DNA of Chlamydomonas sp. SAG25.89, we used the same methods as described for C. euryale. Since no RNAseq data are available for Chlamydomonas sp. SAG25.89, we calculated the theoretical gene model using NCBI Spilign (Kapustin et al. 2008) by aligning the predicted protein for C. euryale to the curated genomic fragments of Chlamydomonas sp. SAG25.89. To calculate the domain structure of the proteins, SMART (Kapustin et al. 2008) was used.

Agarose plug preparation and restriction analysis for Southern blot. Agarose plug protocol (adapted from Sambrook and Russell 2001, Pai et al. 2018). For each plug, 5 x 10^6 cells of either Chlamydomonas sp. SAG25.89 wild type or two of the transgenic lines were harvested from 10-d-old cultures. Cells were centrifuged for 10 min at 4000 g, washed twice with distilled water, and resuspended in 500 µL of distilled water. The final volume of each suspension (about 600 µL to 650 µL) was transferred to a 1.5 mL Eppendorf tube, prewarmed for 5 min at 42°C, mixed with 500 µL of 2% low melting point agarose (SeaKem™, FMC Bioproducts) giving a final cell amount of roughly 10^9 cells · plug^(-1) equivalent to about 10 µg gDNA · plug^(-1). The plugs were let to solidify at 4°C for 15 min.

Afterward, the plugs were transferred to a 50 mL Falcon tube containing 10 mL of TLB buffer (100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, 0.5% [v/v] SDS, pH 8.0). RNAseq A (20 µg · mL^(-1)) was added freshly just before use and incubated for 30 min at 37°C without shaking. Thereafter, 1 mg of Proteinase K was added, and the plugs were incubated overnight at 50°C without shaking. The supernatant was poured off and the plugs were washed twice for 1 h with 10 mL Agarose embedded DNA wash buffer (20 mM Tris-HCl, 50 mM EDTA; pH 7.2). The plugs were then stored in Agarose embedded DNA wash buffer at 4°C until further use.

Individual plugs were transferred to 2 mL Eppendorf tubes and washed twice for 15 min at 4°C with 200 µL 1X New England Biolabs (NEB) CutSmart buffer; then, 200 µL of fresh 1X NEB CutSmart buffer was added and incubated at 4°C overnight. The 1X NEB CutSmart buffer was removed, and 100 µL of fresh 1X NEB CutSmart buffer was added. For each Eppendorf tube, 20 U of the desired restriction enzyme was added, flicked gently to evenly distribute it, and was incubated overnight at 37°C.

Southern blot analysis. The previously prepared agarose plugs were cut in four pieces. One piece was loaded into a well of a 0.3% Megabase certified agarose (Bio-Rad), sealed with 1% low melting point agarose, and run for 1.5–2 h at 3.5 V/cm.

As probes, 300 bp long fragments of the Act1 gene from Chlamydomonas sp. SAG25.89 and the Aph7” CDS from the plasmid were synthesized using the Roche PCR DIG Probe Synthesis Kit according to the manufacturer instructions. As template for the probe synthesis 10 pg of purified Sbf-restricted fragment of pDCF4 and 10 pg of purified PCR-product from the Act1 gene were used. The sequences of the primers used to synthesize the probes and to amplify the fragment from the gDNA (in the case of Act1) were as follows:

OMM2504-Act1_SProbeFw, ATGGCAGAAGAAGGCGCAAG.
OMM2505-Act1_SProbeRv, ATCCTAAATCTAGACACACCCAC.
OMM2502-Aph7”_SProbeFw, GCCCTACCTGTTGATGAG.
OMM2503-Aph7”_SProbeRv, TCCACGAGATGTTGCTCC.

Hybridization and detection were done following the "DIG Application Manual for Filter Hybridization" from Roche using Roche’s Anti-Digoxigenin-AP Fab fragments and Applied Biosystems’ Tropix™ Ready-to-use CDP-Star as substrate.

pH tolerance. Cells were grown in medium NH4-BBM as described before with the following modifications. The buffer of the medium was replaced by a combination of MES, HEPES, and TRIS, each at a final concentration of 20 mM, and the pH was adjusted to the desired value using HCl and NaOH, respectively. The cells were grown for 10 days, and the number of cells was counted at the time of inoculation time and after 10 d. The resulting growth rate was calculated.

Salinity tolerance. Cells were grown in the NH4-BBM medium as described before with the following modifications. Additional NaCl was added to the medium, the concentration of which varied between 0 and 50 g · L^(-1) in intervals of 10 g · L^(-1). The cells were grown for 10 d, and the number of cells was counted at inoculation time and final time. The resulting growth rate was calculated.

Quantitative reverse transcriptase PCR (RT-qPCR). Cells were grown in a 12:12 h light:dark cycle as described above and harvested at LD 3-4. The RNA extraction was performed using the Qiagen RNeasy Plant Mini kit and following the manufacturer’s instructions with an initial biomass of 100 mg algal cells obtained from a 10-d-old culture of each strain. The RT-qPCR was performed using QIAGEN OneStep RT-PCR Kit according to the manufacturer’s instructions, using 7.8 µL of the extracted RNA in a 20 µL reaction volume. The primers used to amplify the Tubulin genes were

Tub-Rv, CTCCAGGTCCATCAGGATGCGAGGAGAC.
Tub1-Fw, ATGGGCACCCAGCCATCATTGCGGCG.
Tub2-Fw, ATGGACCCACGGTTAATTCCATCAGGGCCC.

The primers used to amplify the Act1 gene were

Act1-Fw, CCACACGTGTTTTCACAGAGTGCGGCGTGC and Act1-Rv, GGAATCCAGCACATACATTGCGGAGGAGGAGG.

The cycling parameters were a two-step cycle as described in the kit.
Electroporation using NEPA21 (Yamano et al. 2013). A 7- to 10-d-old culture (50 mL at 1–5 x 10^7 cells ⋅ mL^-1) of *Chlamydomonas* sp., SAG25.89 was pelleted at 4000 g and resuspended twice in sterile milliQ water to wash out all the salts of the medium and lower the conductivity. The cells were pelleted again and resuspended in either NH_4-BBM + 40 mM sucrose (replicates 1 to 3) or in MAX Efficiency™ Transformation Reagent for Algae (Invitrogen, A24229; replicate 4) to a final concentration of 10^6 cells ⋅ mL^-1. For each transformation, an aliquot of 500 μL of cell suspension was transferred to a 1 mL Eppendorf tube and was supplemented with the DNA to be transformed to a final concentration of 10 ng ⋅ μL^-1. To be able to proceed with the electroproporation, the impedance of the sample must range between 400 and 500 Ω. To achieve that value, an initial volume of 90 μL of the cell mixture was applied to the electroporation cuvette and the volume was increased in steps of 20 μL until the right impedance was achieved. The volumes needed oscillated between 90 μL and 400 μL. Detailed parameters for the transformation are listed in Table 1. After electroporation, the cells were resuspended in 10 mL NH_4-BBM including 40 mM sucrose, transferred to a 15 mL Falcon tube, and incubated under dim light (20 μmol ⋅ m^-2 ⋅ s^-1) overnight on a rotary shaker at 23°C. The next day, cells were pelleted, resuspended in 100 μL NH_4-BBM, and plated onto an NH_4-BBM agar plate containing 200 μg ⋅ mL^-1 hygromycin. It should be noted that it is very important to use plates without additional NaCl as high concentrations of NaCl cancel the effect of hygromycin. Transgenic lines grew after four to six weeks.

**Accession numbers.** The *Chlamydomonas euryale* CCMP219 genome sequencing project is available under PRJEB38421, and the *Chlamydomonas* sp. SAG25.89 genome sequencing project under PRJEB38422 at the European Nucleotide Archive (ENA). The following gene assemblies are available under LR798800, *Chlamydomonas* sp. SAG25.89 *Musashi* gene; LR798801, *Chlamydomonas* sp. SAG25.89 *C1* gene; LR798802, *Chlamydomonas* sp. SAG25.89 *Xrn1* gene; LR798803, *Chlamydomonas* sp. SAG25.89 *45S pre-rRNA* gene; LR798804, *Chlamydomonas* sp. SAG25.89 Act1 gene; LR798805, *Chlamydomonas* sp. SAG25.89 Tub1 and Tub2 genes; LR798807, *Chlamydomonas euryale* 45S pre-rRNA gene; LR798808, *Chlamydomonas* sp. SAG25.89 C3 gene.

**RESULTS AND DISCUSSION**

**Phylogeny of the marine green algae *Chlamydomonas* sp. SAG25.89 and *Chlamydomonas euryale*.** To establish a model that would allow the study of biotic interactions between marine motile green algae and other microorganisms and in which the mechanisms and mode of action could be compared with their freshwater counterparts, we evaluated the previously used marine biflagellated alga *Chlamydomonas* sp. SAG25.89 (Aiyar et al. 2017). Its close relative *C. euryale* was also considered due to the availability of its transcriptome ([iMicrobiome assembly MMETSP0063; Keeling et al. 2014](#). *Chlamydomonas* sp. SAG25.89 is smaller (3–4 μm of diameter) than *C. reinhardtii* SAG73.72 (10 μm of diameter) but similar in shape (Fig. 1a). A pyrenoid-like structure can be seen (red arrow in Fig. 1a, middle and right panel). *Chlamydomonas* sp. SAG25.89 corresponds to the strain CCMP235, which was verified with a pairwise alignment of their 18S and 26S rRNA genes, which completely matched the known sequences of *Chlamydomonas* sp. CCMP235 (Accession no. DQ009754 and DQ015721, respectively; Fig. S1a). *Chlamydomonas* sp. SAG25.89 is closely related to *C. euryale* (only one nucleotide difference in the 18S rRNA gene and eight in the entire 26S rRNA gene; Fig. 1b and S1b) and other marine *Chlamydomonas* species from the Moewusinia clade (Watanabe and Lewis 2017). It should be noted that the Moewusinia clade is not solely a marine clade (i.e., *C. moewusii* is a freshwater/soil microalga) but rather contains populations adapted to marine environments, which cluster within this clade. The marine species of the Moewusinia clade are relatively distant from *C. reinhardtii*, which belongs to the Reinhardtinia clade (Lemieux et al. 2015); in total, eight Insertions/Deletions (InDel) and 132 nucleotide substitutions accumulate in the 18S rRNA gene alone comparing *Chlamydomonas* sp. SAG25.89 and *C. reinhardtii* (Aiyar et al. 2017). The close relation of *Chlamydomonas* sp. SAG25.89 and *C. euryale* offers the possibility to use the existing transcriptome data from *C. euryale* to facilitate the elucidation of interesting genes from *Chlamydomonas* sp. SAG25.89. Although we used *C. euryale* for comparison (see

**Table 1. Parameters for the NEPA21-based electroporation for nuclear transformation of *Chlamydomonas* sp. SAG25.89**

| Parameter            | Value   |
|----------------------|---------|
| Sample impedance     | 400–500 Ω |
| Poring pulse         |         |
| Voltage              | 300 V   |
| Pulse Length         | 8 ms    |
| Pulse interval       | 50 ms   |
| Number of pulses     | 1       |
| Decay rate           | 40%     |
| Polarity             | +/-     |
| Transfer Pulses      |         |
| Voltage              | 20 V    |
| Pulse Length         | 50 ms   |
| Pulse interval       | 50 ms   |
| Number of pulses     | 5       |
| Decay rate           | 40%     |
| Polarity             | +/-     |

**Fig. 1.** Light microscopy pictures of *Chlamydomonas* sp. SAG25.89 and its phylogeny. (A) Light microscopy pictures of *Chlamydomonas* sp. SAG25.89 after 10 d of growth in NH_4-BBM + 25 g ⋅ L^-1 NaCl. Left panel, overview of *Chlamydomonas* sp. SAG25.89 cells; middle and right panel, exemplary single cells embedded in 1% low melting agarose and fixed with 0.2% formaldehyde taken as color (middle panel) or black-white picture (left panel). (B) Phylogeny of the 18S rRNA gene of *Chlamydomonas* species and related algae calculated by using the Maximum Likelihood method and General Time Reversible model. The percentage of trees after 1,000 bootstraps in which the associated taxa clustered together is shown next to the branches. The true marine *Chlamydomonas* (labeled in red color), apart from the marine *Chlamydomonas* sp. CCMP681, seem to belong to the Moewusinia clade (Watanabe and Lewis 2017), while the freshwater strains group in both, the Moewusinia and the Reinhardtinia clade (Lemieux et al. 2015). Sequences are presented in Appendix S1. SNP: single nucleotide polymorphism; InDel: Insertions and/or Deletions.
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below), we focused primarily on *Chlamydomonas* sp. SAG25.89 as *C. euryale* was not as motile and, therefore, could not be used in the Orfamide A motility tests to study certain biotic interactions (Aiyar et al. 2017).

We also included other microalgae that are either (i) relevant from an industrial/pharmaceutical point of view (e.g., *Haematococcus pluvialis*), (ii) are used as marine unicellular model organisms (e.g., *Ostreococcus tauri, Micromonas pusilla*), or (iii) for their presence in hypersaline waters (*D. salina*) to provide an overview of the relatedness of these models to *Chlamydomonas reinhardtii* and *Chlamydomonas* sp. SAG25.89. We used the most distant clade as the outgroup (*O. tauri* and *M. pusilla*).

**Environmental factors influencing the growth of Chlamydomonas sp. SAG25.89.** As mentioned in the Introduction, *Chlamydomonas* sp. SAG25.89 was found in the waters of Nantucket Sound (USA) and *C. euryale* in the Yellow Sea (China). First, we analyzed their growth rates under different salt concentrations, where a concentration of 30–32 g·L⁻¹ NaCl (513–547 mM) is typical for the sites where both were isolated (Lanbu et al. 1986, Conant, 2006). We used *C. reinhardtii* for comparison as a typical freshwater alga, which can only tolerate with difficulties low salinities up to about 10 g·L⁻¹ NaCl (171 mM; Fig. 2a), although it is capable to adapt to these low concentrations (specifically 200 mM NaCl, about 11.7 g·L⁻¹ NaCl) and to recover its normal growth rate after several generations (Perrineau et al. 2014). *Chlamydomonas euryale*, on the other hand, shows optimal growth between 10 to 30 g·L⁻¹ NaCl. These concentrations are also within the tolerance range of *Chlamydomonas* sp. SAG25.89 (Fig. 2a). However, *Chlamydomonas* sp. SAG25.89 has a broader salinity tolerance, which ranges from very high salt concentrations (up to 50 g·L⁻¹ NaCl; 856 mM) to freshwater medium (0.025 g·L⁻¹ NaCl, close to 0.43 mM) in which it also grows efficiently. Therefore, this alga is extremely flexible regarding the salt concentration, which was very advantageous for the establishment of a transformation protocol (see below). This also allows for the opportunity to use *Chlamydomonas* sp. SAG25.89 as a model for studies in osmotolerance. In terms of growth at different pH values, both marine strains (Fig. S2a in the Supporting Information), as well as *C. reinhardtii*, tolerate a broad spectrum ranging from pH 5 to pH 9 (Messerli et al. 2005).

We also tested whether *Chlamydomonas* sp. SAG25.89 could grow on acetate like *C. reinhardtii* in mixotrophic growth (light-dark cycle) as well as under constant darkness (heterotrophic growth; Fig. S2b). There was no difference in mixotrophic growth rates with acetate compared to autotrophic growth in a light-dark cycle (16:8 h light:dark). Under heterotrophic growth conditions, *Chlamydomonas* sp. SAG25.89 was not able to grow. Thus, *Chlamydomonas* sp. SAG25.89 cannot use acetate as a carbon source, so far due to unknown reasons, in contrast to *C. reinhardtii*.

We assessed different nitrogen sources for the growth of *Chlamydomonas* sp. SAG25.89, such as nitrate, nitrite, and ammonia. Interestingly, it could only grow on ammonia, the reduced nitrogen source (Fig. 2b). Using the long-read PacBio sequencing data (see Methods), we assembled a preliminary draft of the genome of *Chlamydomonas* sp. SAG25.89 and also of the genome of *C. euryale*. We assembled 699,427 (*C. euryale*) and 450,484 (*Chlamydomonas* sp. SAG25.89) reads per strain with a mean read length of 6,560 bp and 7,782 bp into draft assemblies comprising 745 and 403 contigs (4,392 and 2,417 unitigs), respectively. The largest contigs reached a size of 845 kbp (*C. euryale*) and 1,8 Mbp (*Chlamydomonas* sp. SAG25.89). Even without additional polishing steps, these drafts were of sufficient quality to assess the general genome structure and synteny of specific gene clusters in this study.

Using the genome draft of *Chlamydomonas* sp. SAG25.89, we were able to analyze potential reasons for the lack of growth in oxidized nitrogen sources. Notably, we could not find any gene sequences encoding neither the nitrate reductase (*Nia1*), nor the nitrite reductase (*Nii1*), nor the transporters for nitrate and nitrite in the plasma membrane (Fig. 2c, Fig. S3 in the Supporting Information). In contrast, the gene sequences encoding the ammonium transporters (*Amt* family) and glutamine synthetases (*Gln* family) were present. In *C. reinhardtii*, randomly occurring single nucleotide mutations in the *Nii1* gene lead to a loss of its activity. Although the gene is still present, these algal strains cannot use nitrate

![Fig. 2](image-url). Growth of *Chlamydomonas* sp. SAG25.89 under different conditions in a light:dark 16:8 h cycle. (A) Growth of *C. reinhardtii*. *Chlamydomonas* sp. SAG25.89 and *C. euryale* at different NaCl concentrations ranging from freshwater (0 g·L⁻¹ NaCl) to hypersaline waters (50 g·L⁻¹). (B) Growth rates of *Chlamydomonas* sp. SAG25.89 in the presence of different nitrogen sources. (A, B) For each experiment, nine biological replicates were performed. Error bars represent standard deviation (SD). (C) Schematic of genes/proteins for the nitrogen metabolism present in *C. reinhardtii* including transporters (Nrt, Nar, Amt) in the plasma and chloroplast membranes as well as Nitrate reductase (*Nia*), Nitrite reductase (*Nii*), and glutamine synthetases (*Gln*; Schmollinger et al. 2014, Sanz-Luque et al. 2015). Conserved gene sequences in *Chlamydomonas* sp. SAG25.89 are shown in blue, missing ones are indicated in gray. (D) Growth of *Chlamydomonas* sp. SAG25.89 at different temperatures. For each experiment, four biological replicates were performed. Error bars represent SD. (E) Predicted *Chlamydomonas* sp. SAG25.89 proteins with conserved domains (in green) that are known to be involved in temperature integration and/or acclimation in *C. reinhardtii* (Voytsekh et al. 2008, Li et al. 2018). The presence of several Met and Gly residues after the second RRM domain, as present in the ELAV-like family (CELF) protein G3 (Zhao et al. 2004) or Musashi (Li et al. 2018), is indicated in yellow. RRM, RNA recognition domain; KH, K homology; WW, protein interaction domain.
(Merchant et al. 2007). To investigate further whether the marine *Chlamydomonas* species may have lost some of the genes involved in nitrogen metabolism, we used publicly available genome and transcriptome data from all the available *Chlamydomonas* strains. We also assembled a preliminary draft of the *C. euryale* genome as mentioned above, which was used in conjunction. Interestingly, we were unable to find any signs of the usual nitrate gene cluster (Fernandez and Galvan 2008) in either *Chlamydomonas* sp. or *C. euryale*. *Chlamydomonas* sp., *C. euryale* and some other species lacked all or some of the genes encoding nitrate and nitrite transporters as well as *Nia1* and *NiI* (Fig. S3).

In the marine model picocysta *Ostrococcus tauri* and in *Micromonas pusilla*, however, the nitrate gene clusters are present (Sanz-Luque et al. 2015). These data suggest that some marine green *Chlamydomonas* algae may have lost these genes completely.

We were also interested in finding out whether the growth of *Chlamydomonas* sp. SAG25.89 changed at different temperatures. The average temperature in the ocean is approximately 17°C, ranging from 27 to 30°C near the equator to below the freezing point (−2°C) within the polar region (Banfalvi 2016). At Nantucket Sound, where *Chlamydomonas* sp. SAG25.89 was isolated, the average temperatures are 23°C in summer and 10°C in winter (Conant, 2006). We chose a range of 15°C, 18°C, 23°C, 28°C, and 32°C to cover the average spectrum and to examine at which temperature the cells may become heat stressed. *Chlamydomonas* sp. SAG25.89 grew well and at a similar rate in a light-dark cycle of 16:8 h from 15°C to 28°C with a slight preference for 23°C (Fig. 2d). It reaches its stationary phase after 12 d before it starts its decline phase (Fig. 2d). At 32°C, *Chlamydomonas* sp. SAG25.89 cannot grow; this temperature is obviously already in the heat stress range for this alga. These data suggest that the heat stress temperature is lower for *Chlamydomonas* sp. SAG25.89 compared to *Chlamydomonas* reinhardtii (Tanaka et al. 2000, Rüdiger et al. 2017). In *Chlamydomonas reinhardtii*, some molecular components that mediate thermal acclimation at temperatures between 18°C and 28°C are known (Zhao et al. 2004, Voytsekh et al. 2008, Li et al. 2018). These include the C1 and C3 subunits of the RNA-binding protein CHLAMY1, the 5′-3′ endoribonuclease Xrn1 as well as the RNA-binding protein Musashi, which is present in various splicing variants. These components are either expressed at different levels in cells grown between 18°C and 28°C or their phosphorylation pattern changes (in case of C1). C1, C3, and XRN1 are also known members of the circadian clock in *C. reinhardtii* (Iliev et al. 2006, Dathe et al. 2012). The knock-down, knock-out (C3 and Xrn1), or overexpression (Musashi) of some of these components alter the algal temperature-dependent growth rates, suggesting that they confer thermal acclimation in addition to their clock function (Li et al. 2018). The genes encoding these subunits seem to be conserved in *Chlamydomonas* sp. SAG25.89 (Fig. 2e); their typical domains are conserved well enough to be recognized by detection software, but so far we have no functional data in *Chlamydomonas* sp. SAG25.89. Thus, the C1 subunit carries two of three KH domains responsible for RNA binding and the WW domain (protein interaction domain) at its C-terminus (Fig. 2e; Zhao et al. 2004). In the case of C3, all domains are conserved. Three RRM (RNA recognition domains) are present and the second and third are separated by a Met-rich spacer (Zhao et al. 2004). Musashi, having several splicing variants in *C. reinhardtii*, resembles some of them with three RRM domains (Li et al. 2018). This provides a well-defined basis for further molecular studies and potential involvement in thermal acclimation in the marine *Chlamydomonas* sp. SAG25.89.

Characterization of *Chlamydomonas* sp. SAG25.89 antibiotic resistances and cis-acting elements as basis for transformation. Our main goal was to establish a transformation protocol for *Chlamydomonas* sp. SAG25.89, which would allow for the introduction of diverse reporter genes in the future and performance of functional studies on genes of this marine alga (e.g., by gene silencing or genome editing). For this purpose, several important features had to be evaluated. First, it was necessary to find a selection marker. Therefore, common antibiotics were checked at different concentrations (Table 2). Although *Chlamydomonas* sp. SAG25.89 showed growth with kanamycin, streptomycin, and neomycin up to a relatively high concentration (50–200 µg · mL⁻¹); so far for unknown reasons, in contrast to *C. reinhardtii*, it could not grow at concentrations of paromomycin and hygromycin equal or higher than 50 µg · mL⁻¹. We chose the hygromycin conferring resistance gene *Aph7* as the selection marker for the vector because the established aequorin reporter for *C. reinhardtii* (Aiyar et al. 2017) is based on this resistance and we want to use parts of this vector also for *Chlamydomonas* sp. SAG25.89 in the future.

| Antibiotic concentration (µg · mL⁻¹) | 0 | 0.5 | 1 | 5 | 10 | 50 | 100 | 200 |
|-------------------------------------|---|-----|---|---|----|----|-----|-----|
| Kanamycin                           | + | +   | +  | + | +  | +  | +   | +   |
| Paromomycin                         | + | +   | +  | + | +  | -  | -   | -   |
| Streptomycin                        | + | +   | +  | + | +  | +  | +   | +   |
| Neomycin                            | + | +   | +  | + | +  | +  | -   | -   |
| Hygromycin                          | + | +   | +  | + | +  | +  | -   | -   |

+ Algal cells grow on the NH₄-BBM medium; - no algal growth.
The vector has to contain the right GC content as well as codon-compatible sequences so that it can be properly expressed by the host. Most Chlamydomonas species have a high GC content (>60%) including C. euryale and Chlamydomonas sp. SAG25.89 (Table S2 in the Supporting Information) and their freshwater counterpart C. reinhardtii (Merchant et al. 2007). Since the transcriptome of C. euryale was available and this marine species is related closely to Chlamydomonas sp. SAG25.89, we used it to calculate the codon usage. The codon usage of C. euryale and C. reinhardtii is very similar in most cases; both share the same preferred codons (Fig. 3a). Because C. euryale and Chlamydomonas sp. SAG25.89 are even closer relatives than C. euryale and C. reinhardtii (Fig. 1b), we expect that this also accounts for Chlamydomonas sp. SAG25.89. This offers the unique opportunity to use genes established already for C. reinhardtii in Chlamydomonas sp. SAG25.89 as well.

For the successful transformation of Chlamydomonas sp. SAG25.89, the coding sequence of the Aph7'' gene conferring resistance to hygromycin was used, as established for C. reinhardtii in the vector pHyg4 (Berthold et al. 2002). It contains the native sequence of the Aminoglycoside phosphotransferase (Aph7") gene from Streptomyces hygroscopicus, which is also GC-rich (70.8%), has a similar codon usage to C. reinhardtii, and contains an additional stop codon at the 3' terminus with the most frequent stop codon of C. reinhardtii to guarantee the proper stop of transcription (Berthold et al. 2002).

Moreover, we evaluated the polyadenylation signal present before the start of the poly(A) tail in the transcriptome of Chlamydomonas euryale. The preferent poly(A) signal found was TG(C/T)AA (Fig. 3b), which matches the most dominant poly(A) signal present in ~50% of the genes of C. reinhardtii (TGTA; Zhao et al. 2014, Bell et al. 2016). The TG (C/T)AA poly(A) signal is also present in the β-
Tubulin (Tub1 and Tub2) and the Act1 genes of Chlamydomonas sp. SAG25.89 that were selected for further analysis.

Members of the cytoskeleton, such as tubulin and actin, are usually expressed strongly, often in a constitutive manner, as they are essential for many cellular processes and/or flagellar structure. Thus, we looked for the Tub1, Tub2, and Act1 genes in Chlamydomonas sp. SAG25.89. Our aim was to take the cis-acting elements including the promoter as well as the 5'- and 3'-UTRs from one of these genes to ensure good expression levels of the hygromycin.

FIG. 4. Search for cis-acting elements for Chlamydomonas sp. SAG25.89. (A) Arrangement of the β-Tubulin genes (Tub1 and Tub2) of Chlamydomonas sp. SAG25.89 compared to C. reinhardtii showing the significant shortening of the spacer region between them down to 255 bp in Chlamydomonas sp. SAG25.89. (B) Intron (light gray lines)/exon (dark gray boxes) sequence conservation between the Actin (Act1) genes and their flanking regions (lighter gray bars up- and downstream) of C. reinhardtii and Chlamydomonas sp. SAG25.89. (A, B) The genomic organization of the Tub1, Tub2, and Act1 genes result from the analysis of the Chlamydomonas sp. SAG25.89 genome sequence and the according cDNAs from C. euryale. (C) Relative expression of the Tub and Act genes normalized with the Act1 gene expression. Growth conditions for qPCRs are described in Methods. For the experiment, three biological replicates with three technical replicates each were performed. Error bars represent SD. A two-tailed unpaired Student’s t-test was done. *P < 0.05 indicates a statistically significant difference.

FIG. 5. Design of a vector for stable transformation of Chlamydomonas sp. SAG25.89. (A) Structure of Chlamydomonas sp. SAG25.89 transformation vector pDCF4 containing the hygromycin expression cassette along with the endogenous Chlamydomonas sp. SAG25.89 Act1 promoter and its 5'- and 3'-untranslated regions (UTRs) presented in gray, the Aph7" coding sequence (purple), and the plasmid replication and selection system for E. coli with the origin of replication (yellow), the Kanamycin resistance (green), and the Ampicillin promoter (white). The enzymes used to linearize the plasmid for transformation in Chlamydomonas sp. SAG25.89 are depicted in red and the qPCR primers used for bulk screening are depicted in blue. (B) Overview of the transformation protocol. (C) Pictures of transgenic lines and controls for the transformation of Chlamydomonas sp. SAG25.89 using pDCF4 on media with (+Hyg) and without (-Hyg) hygromycin. Colonies were picked and transferred to a new plate as soon as they were visible to avoid merging. The pictures were taken after further incubation of the original plate once the (already picked) colonies were big enough to be seen in the pictures. (D) Verification of nuclear integration of the Aph7" cassette using the qPCR primers for bulk screening. A fragment of 113 bp can be seen for the positive control with vector DNA (pDCF4) as well as for the transgenic lines (TL) but is missing in wild type (WT) and in the no template control (NT). (E) Southern Blot analysis with an Act1 probe in WT, TL2, and TL4. Genomic DNAs were restricted with the indicated enzymes as described in Methods. (F) Southern Blot analysis with an Aph" probe in WT, TL2, and TL4. Genomic DNAs were restricted with the indicated enzymes as described in Methods. (E, F). The used membrane was the same for both probes; it was stripped in between and rehybridized.
GENETIC TOOLS IN CHLAMYDOMONAS SP.

A

--- Diagram of genetic tools in Chlamydomonas sp. ---

B

- Culture algae
  - Day/night cycle
  - Temperature
  - Salt concentration
  - Time
  - Centrifugation speed
  - Cell concentration
  - DNA concentration
  - Volume
  - Antibiotic concentration

- Centrifuge & wash
  - 4,000g
  - 4°C
  - 0 g L⁻¹
  - 10 min

- Resuspend
  - 10⁶ cell mL⁻¹
  - 4°C
  - 0 g L⁻¹

- Prepare sample
  - 10 ng μL⁻¹
  - RT
  - 0 g L⁻¹
  - 0.4-0.5 Ω

C

- Positive control
  - -pDCF4 / - Hyg

- Negative control
  - -pDCF4 / + Hyg

- Transgenic lines
  - +pDCF4 / + Hyg

D

- Radiogram showing banding patterns
  - 200 bp
  - 100 bp

E

- Gel electrophoresis
  - kbp
  - WT
  - pDCF4
  - TL #1
  - TL #2
  - TL #3
  - TL #4

F

- DNA fragment analysis
  - kbp
  - Styl
  - Msel
  - PstI
  - Ncol

--- Diagram of genetic tools in Chlamydomonas sp. ---
selection marker *Aph7*" to create a highly efficient vector. In *C. reinhardtii*, there are two β-Tubulin genes (Tub1 and Tub2; Youngblom et al. 1984), which are located in chromosome 12 ~ 0.8 Mbp apart (Cre12.g542250 and Cre12.g549550, respectively; Fig. 4a). In *Chlamydomonas* sp. SAG25.89, there are also two β-Tub genes, but they are separated only by 255 bp (Fig. 4a), making it challenging to select and distinguish the promoters and UTRs that do not overlap with the other gene, which could lead to problems with expression. The Act1 gene was present in both organisms as a single copy gene. The structure of the exons/introns varied slightly (Fig. 4b). To determine whether the Tubulin genes were expressed at different levels than Act1, we performed RT-qPCR. We found that Act1 is expressed at a slightly higher rate than Tub1 and Tub2 (Fig. 4c). For all reasons mentioned above, we focused on Act1.

Establishment of a transformation vector and protocol for Chlamydomonas sp. SAG25.89 stable nuclear transformation. We constructed the transformation vector pDCF4 (Fig. 5a) for amplification in *E. coli* and expression in *Chlamydomonas* sp. SAG25.89 (see sequence in Fig. S4 in the Supporting Information). In addition to *E. coli* specific features, it contains 666 bp of the *Chlamydomonas* sp. SAG25.89 Act1 promoter and 5'-UTR upstream of the *Aph7"* coding sequence and 1,386 bp of the potential Act1 3'-UTR downstream, including all possible polyadenylation signals (Fig. 5a). The vector was transformed in three independent experiments either as a circular plasmid or linearized with the restriction enzyme *AgeI*, which cuts within the *Kanamycin* resistance gene for *E. coli*. Moreover, the vector was restricted with *SfiI*, which has two restriction sites that precisely flank the *Aph7"* cassette (Fig. 5a), and the resulting *Aph7"* cassette fragment was also transformed. For the transformation, an electroporation protocol using the NEPA 21 electroporator was established, which allows for the adjustment of numerous parameters necessary for optimizing the transformation of algal cells (Yamano et al. 2013). The transformation buffer must lack NaCl for the electroporation process to be successful. We took approximately six to eight weeks for successfully transformed cells to form colonies on hygromycin NH₄-BBM agar plates (Fig. 5c).

All transformations were performed at the same ratio of 10 ng DNA per 10⁵ algal cells. The linearized vector achieved a significantly higher yield than circularized plasmid DNA (Table 3). The results with the linearized plasmid using either *AgeI* or *SfiI* were similar (Table 3). The transgenic lines were also grown successively on medium containing hygromycin and, thereafter, on medium lacking hygromycin and again on medium with hygromycin. The growth was consistent in all cases.

In addition to hygromycin resistance, the integration of the cassette into the genome of *Chlamydo- monas* sp. SAG25.89 was verified through PCR using some of the transgenic lines with primers amplifying 113 bp of the 3’ terminus of the *Aph7"* coding sequence (Fig. 5a) after at least two rounds of restreaking the colonies before use in colony PCR (Fig. 5d).

Moreover, Southern blots were performed to check for the integration of the cassette into the genome (Fig. 5). As a positive control, we used the Act1 gene (Fig. 5e). The genomic DNAs of wild type and the transgenic lines 2 and 4 were restricted with *Styl, MseI*, *Neol*, and *PstI* and probed with labeled Act1 (see Methods). The calculated sizes of 1.64 kbp for *Styl*, 3.5 kbp for *MseI*, 4.5 kbp for *PstI*, and 5.2 kbp for *Neol* were visible in all cases (Fig. 5e). In transgenic line 2, a second higher kbp band was also visible in case of *PstI*, which is likely due to partial restriction as it does not appear in wild type and in transgenic line 4. The size of this band is in accordance with the size (8.4 kbp) of a skipped *PstI* restriction site in the genome. Also, in case of *MseI*, a second higher and weaker band was visible in all cases in accordance with a skipped site. The genomic DNAs of wild type and the mentioned two transgenic lines were also used for labeling with the *Aph7"* probe (see Methods). All enzymes used (*Styl, MseI, Neol*, and *PstI*) do not cut within the *Aph7"* or *ActI* probe regions. As expected, no signals were visible in wild type with the *Aph7"* probe (Fig. 5f, left panel). In transgenic line 2 (Fig. 5f, middle panel), two fragments hybridized in case of the *Neol* digest, and more than two for *Styl*, where partial digests may have occurred. These data indicate that the *Aph7"* cassette was integrated more than once in the genome of transgenic line 2. In transgenic line 4 (Fig. 5f, right panel), a single integration event of the *Aph7"* cassette

| Table 3. Transformation efficiency of linearized and circular pDCF4 for Chlamydomonas sp. SAG25.89 |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| **Replica** | **Replica 1** | **Replica 2** | **Replica 3** | **Replica 4** |
| Linearized | 18 | 18 | 11 | 21 |
| Circularized | 1 | 0 | 1 | 0 |

Linearized versions of replica 1 to 3 represent pDCF4 cut with *AgeI*; in the case of replica 4, pDCF4 was cut with *SfiI*. Details about the amount of DNA and used cell numbers are described in Methods.
seems to have taken place as one major band is visible in all cases. These data corroborate that the Aph7" cassette is being integrated into the genome by the established transformation method.

In summary, the vector pDCF4, along with the established transformation protocol, can now be used as a basis for the genetic manipulation of this marine green algal species. As outlined above, reporters used for Chlamydomonas reinhardtii should be easy to transfer to Chlamydomonas sp. SAG25.89 based on the high GC content (Table S2) and similar codon usage, at least in the closely related C. eurystome (Fig. 3a). Thereby, the recently developed MoClo toolkit enabling synthetic biology in C. reinhardtii (Crozet et al. 2018) can serve as an efficient basis for Chlamydomonas sp. SAG25.89.

A long-term plan is to improve the genome sequences of Chlamydomonas sp. SAG25.89 by additional Oxford Nanopore sequencing, which yields larger fragments and, thus, ensures an efficient and more contiguous assembly. Nanopore sequencing should provide reads that extend beyond most of the genomic repeats, allowing an assembly with good contiguity and avoiding chimeras between chromosomes due to long stretches of repetitive and nearly identical sequences. Furthermore, once the contiguity and potential misassemblies have been resolved, the Illumina data can be used to polish it, resulting in a high-quality, high-contiguity genome.

CONCLUSIONS

We developed an efficient electroporation-based transformation protocol for the marine Chlamydomonas sp. SAG25.89, which can now serve as a basis for studies on biotic and abiotic interactions. The data show that the exponential growth of this marine protist stops at 32°C while it grows well in a range from 15°C to 28°C. Some components that confer thermal acclimation in C. reinhardtii are conserved in Chlamydomonas sp. SAG25.89, allowing comparative studies in the future. Due to the high GC content in both C. reinhardtii and Chlamydomonas sp. SAG25.89, the numerous established codon-adapted GC-rich reporters for C. reinhardtii (Salomé and Merchant 2019) are easily transferable to the marine species. The alga will be also amenable to genetic approaches including silencing or gene editing. Our studies also revealed unexpected insights on the physiology (osmotolerance) and the genome of Chlamydomonas sp. SAG25.89 such as the likely loss of some genes of nitrogen metabolism or the translocation of the two Tub genes.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web site:

**Figure S1.** Full alignment of the 45S pre-rRNA cluster of *Chlamydomonas* sp. SAG25.89 with the 18S and 26S rRNA genes of *Chlamydomonas* sp. CCMP235 (A) and differences of the 45S pre-rRNA cluster compared to *C. euryale* (B).

**Figure S2.** pH tolerance of *Chlamydomonas* sp. SAG25.89 and *C. euryale* (A) and comparison of growth rates of *Chlamydomonas* sp. SAG25.89 under photoauto-, mixo- and heterotrophic conditions (B).

**Figure S3.** Presence of different proteins involved in nitrogen metabolism in the different sequenced strains of *Chlamydomonas*.

**Figure S4.** Full sequence of pDCF4.

**Table S1.** Parameters used to run CENTRIMO and DREME.

**Table S2.** Conservation of high GC content in the nuclear genomes of different *Chlamydomonas* species.

**Appendix S1.** Multiple sequence alignment of the 18S rDNA (FASTA file; Fig. 1b).

**Appendix S2.** In-house script for processing the transcriptome and selecting mRNAs containing the poly(A) tail.