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Production of the recombinant spider silk MaSp2 protein using the marine purple photosynthetic nonsulfur bacterium Rhodovulum sulfidophilum under autotrophic conditions

Miki Suzuki[1] and Keiji Numata[1,2]

Abstract

Our research focuses on *Rhodovulum sulfidophilum*, a marine, anoxygenic purple nonsulfur photosynthetic bacterium (PNSB), as a tool for the production of materials for a sustainable society. The most sustainable approach with PNSB is to culture it under autotrophic conditions using carbon dioxide and nitrogen fixation pathways. By introducing exogenous genes of interest, *R. sulfidophilum*, one of the most studied PNSB, can serve as a protein expression host under autotrophic conditions. Herein, through autotrophic culture, recombinant spider silk proteins were successfully produced. This research is expected to contribute to the development of sustainable material production using PNSB.

Introduction

The production of useful substances using light energy by microorganisms with photosynthetic metabolic pathways has attracted attention as an environmentally friendly method[1,2]. Compared to traditional plant-based protein production, it offers advantages such as reduced area requirements, higher cellular protein content, and shorter growth times[1,3]. Furthermore, some of these microorganisms have metabolic pathways for nitrogen fixation in addition to photosynthesis, and unlike plants, they can supply their own nitrogen source, which is necessary for growth. Purple nonsulfur photosynthetic bacteria (PNSB) are attracting attention as such tools for environmentally friendly, resource-circulating societies because they can fix gaseous CO_2 and N_2 in the environment in their metabolic system[3]. PSNB perform anoxygenic photosynthesis but are facultative anaerobes; thus, they do not require strict oxygen-free conditions, making culturing methods simple[3]. Since PNSB are rich in amino acids, they are expected to be used as agricultural fertilizers[4], as bait in fisheries[3] or as hosts for the expression of heterologous proteins via genetic engineering[5,6]. PNSB also accumulates other useful substances, such as carotenoids, vitamins, and biodegradable plastics, depending on the culture conditions[3,6]; thus, they are expected to be useful production tools. By utilizing light energy, PNSB can grow rapidly in mineral medium containing simple organic carbon (C) sources such as butyrate, acetate, or propionate anaerobically[7]. Even in the absence of organic C compounds, by using light energy, PNSB can grow lithoautotrophically[8]: they can fix CO_2 via electrons from reduced inorganic compounds such as H_2S, $Na_2S_2O_3$[8], and metals[9]. PNSB have also been investigated as bioremediation tools because they have a relatively high tolerance to metals and can detoxify toxic metals through oxidation and reduction[9]. Among PNSB, we focus on *Rhodovulum sulfidophilum*, a marine PNSB that exhibits superior reduced inorganic sulfur compound metabolism[8,10,11]. The sulfur concentration in seawater is higher than that in freshwater rivers and lakes[12]; hence, *R. sulfidophilum* possesses a sulfur-oxidizing gene cluster, the *sox* gene[8,10]. In our previous study, little growth of *R. sulfidophilum* was observed when N_2 gas and $NaHCO_3$

Correspondence: Keiji Numata (keiji.numata@riken.jp)
[1]Department of Material Chemistry, Graduate School of Engineering, Katsura Campus, Kyoto University, Nishikyo-ku, Kyoto, Japan
[2]Biomacromolecules Research Team, RIKEN Center for Sustainable Resource Science, Wako, Saitama, Japan

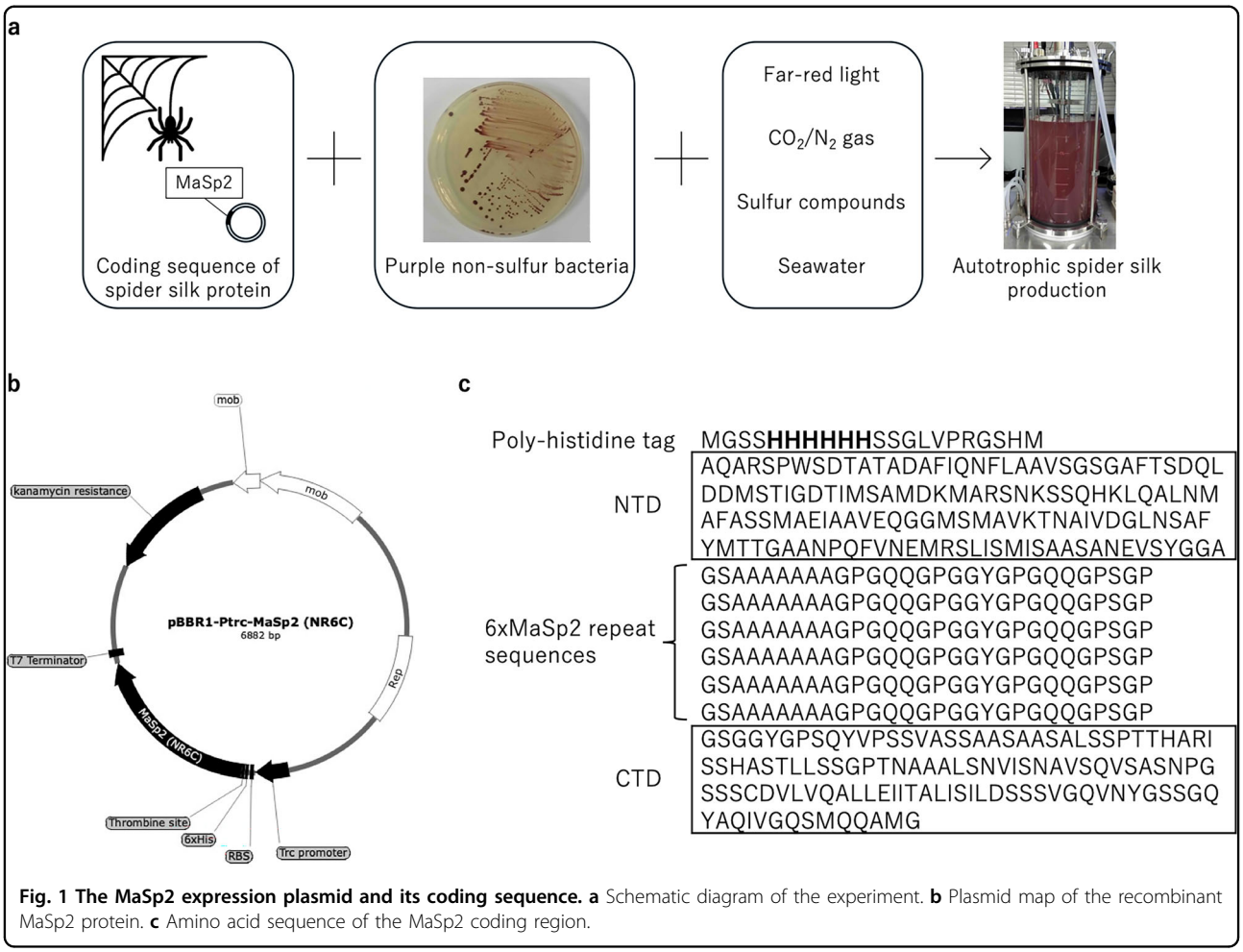

Fig. 1 The MaSp2 expression plasmid and its coding sequence. a Schematic diagram of the experiment. **b** Plasmid map of the recombinant MaSp2 protein. **c** Amino acid sequence of the MaSp2 coding region.

were added as inorganic N and C sources to artificial seawater[5], but the addition of $Na_2S_2O_3$ promoted bacterial growth by inducing not only the CO_2 and nitrogen (N) fixation pathways but also possibly the lithotrophic sulfur oxidation metabolic pathway[13]. When light energy is provided for growth, PNSB exhibit a high growth rate under heterotrophic conditions; therefore, applied research on PNSB has been conducted mainly under photoheterotrophic conditions[3,7], while the most sustainable approach would likely involve culturing bacteria under autotrophic conditions with pathways to fix CO_2- and N, using gaseous CO_2 and N_2 present in large amounts in the air[5]. Furthermore, seawater is inexhaustible and has less impact on the environment than freshwater does, reducing costs and the risk of bacterial contamination due to its high sodium chloride content[13]. Exploiting these culture conditions, we aimed to produce useful heterologous *R. sulfidophilum* proteins for the purpose of sustainable material production (Fig. 1a).

As a recombinant protein produced by *R. sulfidophilum*, we focused on major ampullate spidroin 2 (MaSp2), which is produced by the spider *Trichonephila clavipes*[14,15]. The soluble form of the protein self-assembles into hierarchical structures, generating insoluble fibrous structural proteins by being processed in spider glands. The fibrous protein produced by spiders is commonly called spider silk and has attracted high interest: it is strong and flexible, making it promising for industrial applications in clothing, medical textiles, and other areas[15–18]. Spiders cannot be cultured artificially because of their cannibalism; thus, efforts are underway to use genetic engineering to overexpress soluble silk proteins in microorganisms such as yeast and *Escherichia coli*[15] grown in medium rich in organic carbon and nitrogen sources, after which the collected silk proteins are converted into fibrous forms with original hierarchical structures using organic solvents or aqueous solutions such as phosphate buffer[17,18]. Here, we aimed to produce spider silk protein using an autotrophic process to address sustainability issues in biomanufacturing fields. In our previous paper, recombinant *R. sulfidophilum* harboring a plasmid encoding a different major ampullate spidroin 1 (MaSp1) was cultivated under photoautotrophic conditions[5]. When cultured for 7 days in artificial seawater

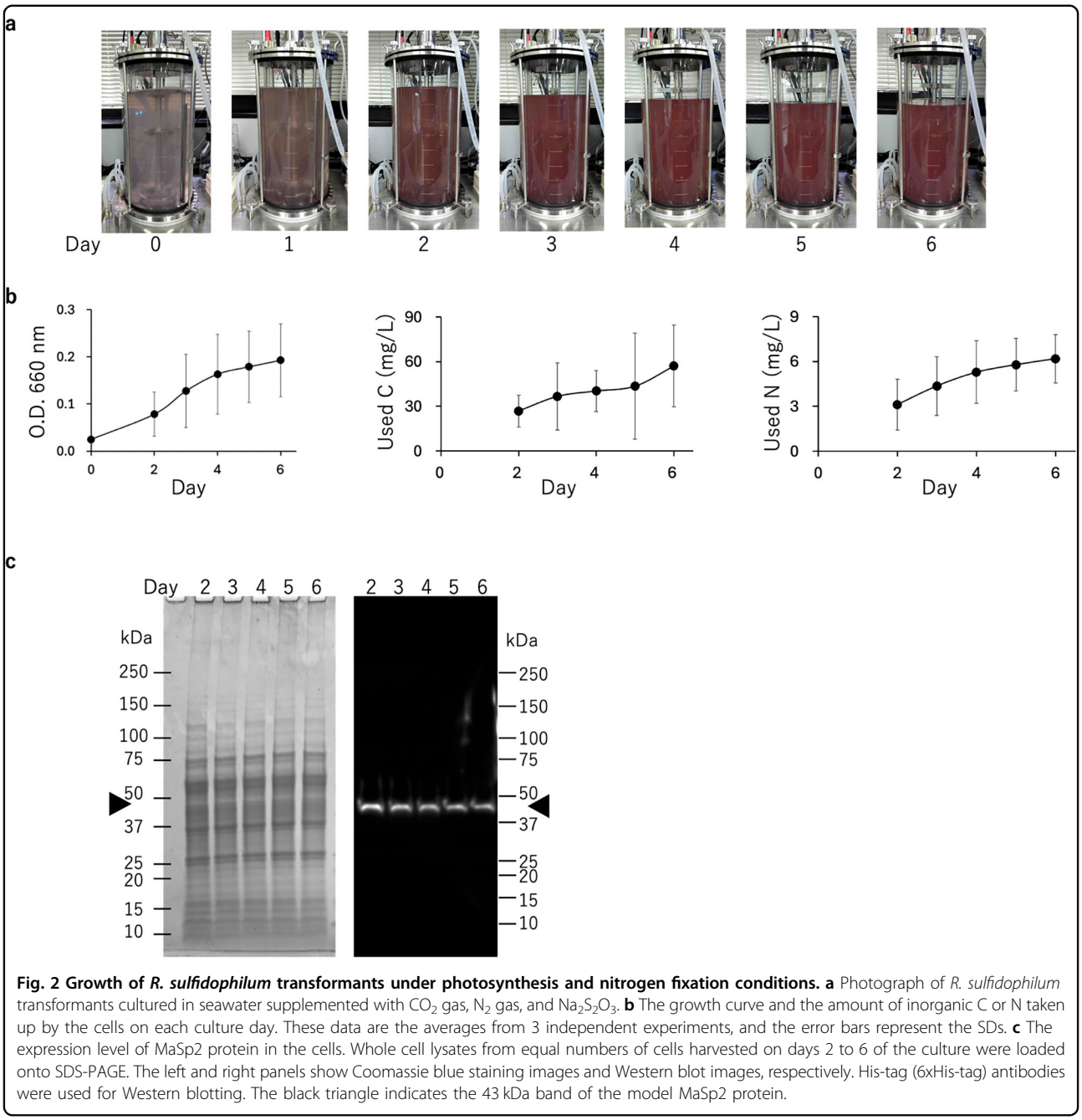

Fig. 2 Growth of *R. sulfidophilum* transformants under photosynthesis and nitrogen fixation conditions. a Photograph of *R. sulfidophilum* transformants cultured in seawater supplemented with CO_2 gas, N_2 gas, and $Na_2S_2O_3$. **b** The growth curve and the amount of inorganic C or N taken up by the cells on each culture day. These data are the averages from 3 independent experiments, and the error bars represent the SDs. **c** The expression level of MaSp2 protein in the cells. Whole cell lysates from equal numbers of cells harvested on days 2 to 6 of the culture were loaded onto SDS-PAGE. The left and right panels show Coomassie blue staining images and Western blot images, respectively. His-tag (6xHis-tag) antibodies were used for Western blotting. The black triangle indicates the 43 kDa band of the model MaSp2 protein.

supplemented with 12 mM $NaHCO_3$ as an inorganic C source and 0.5 L/day of N_2 gas as an N source, the cells showed little growth. In contrast, in this study, recombinant *R. sulfidophilum* harboring a MaSp2 expression plasmid was cultured in artificial seawater supplemented with CO_2 gas as an inorganic C source, N_2 gas as a N source, and a relatively high concentration of $Na_2S_2O_3$ (approximately 9.7 mM) as an electron donor. This promoted cell growth (Fig. 2, Table 1) and resulted in the production of partially purified soluble MaSp2 protein at a yield of 3.2 ± 3.8 μg/L (Table 2).

Results

The addition of inorganic reduced sulfur compounds to artificial seawater increased cell proliferation

To facilitate *R. sulfidophilum* growth on a larger scale in a shorter culture period, in addition to inorganic and organic C/N dissolved in artificial seawater[5], it is necessary to inject gaseous CO_2 and N_2 into seawater. The pH of seawater is slightly alkaline, approximately 8, and at this pH, most CO_2 dissolved in seawater exists as HCO_3^- ions. By injecting gaseous CO_2, the pH of seawater quickly decreases from approximately 8 to approximately 5, with

Table 1 Analysis of cell growth and the amount of inorganic C or N taken up by cells during 6-day culture.

	Absorbance	specific growth rate	Used C	Used N	Used C	Used N	Used C	Used N	C:N ratio
Day	(OD_{660})	(μ/day)	(mg/L)	(mg/L)	(mg/L/OD_{660})	(mg/L/OD_{660})	(mol/L/OD_{660})	(mol/L/OD_{660})	
0	0.025 ± 0.011								
2	0.078 ± 0.047		26.8 ± 10.8	3.1 ± 1.7	373.8 ± 119.2	40.5 ± 1.8	31.1 ± 9.9	2.9 ± 0.1	10.7 ± 3.0
3	0.128 ± 0.078		36.6 ± 22.6	4.4 ± 2.0	287.2 ± 17.0	36.3 ± 6.1	23.9 ± 1.4	2.6 ± 0.4	9.4 ± 1.5
4	0.163 ± 0.085		40.3 ± 13.7	5.3 ± 2.1	261.9 ± 60.9	33.7 ± 4.3	21.8 ± 5.1	2.4 ± 0.3	9.1 ± 2.0
5	0.179 ± 0.076		43.5 ± 35.7	5.8 ± 1.8	218.2 ± 91.8	33.4 ± 3.6	18.2 ± 7.6	2.4 ± 0.3	7.9 ± 4.2
6	0.193 ± 0.077		57.1 ± 27.6	6.2 ± 1.6	290.6 ± 32.8	33.3 ± 4.1	24.2 ± 2.7	2.4 ± 0.3	10.4 ± 2.3
0-2		0.56 ± 0.13							
2-3		0.49 ± 0.15							
3-4		0.28 ± 0.09							
4-5		0.12 ± 0.09							
5-6		0.08 ± 0.03							

Growth of *R. sulfidophilum* transformants (absorbance at 660 nm), the specific growth rate, the amount of inorganic C or N taken up by the cells (measured in the culture medium or in the supernatant obtained by centrifugation of the culture medium) measured using the TOC/TN instrument, and the C:N molar ratio calculated from these values. Each value is the average ± SD of three independent experiments.

CO_2 dissolved in seawater existing in the form of gaseous CO_2 rather than HCO_3^- ions, and the solubility of gaseous CO_2 is low. Thus, during gas injection, 1 N NaOH was continuously added dropwise to dissolve CO_2 in the form of HCO_3^- ions. In a 10 L-capacity incubator filled with 8.9 L of artificial seawater, CO_2 and N_2 gas were bubbled at rates of 0.15 L/min and 0.35 L/min, respectively, for 2 h at 30 °C. Before gas injection, the inorganic C concentration of artificial seawater was approximately 20–22 mg/L, and the 2 h gas injection resulted in approximately 340–420 mg/L inorganic C. After the gas supply was stopped, $Na_2S_2O_3$ was added as an electron donor to a final concentration of 9.7 mM, followed by the addition of an antibiotic solution and a culture of *R. sulfidophilum* transformed with a plasmid encoding the MaSp2 gene (Fig. 1b). The coding region consisted of the three parts, N-terminal domain (NTD) and C-terminal domain (CTD), with a core 6 tandem repeat sequence between them, mimicking the structure found in nature (Fig. 1c). Because the core tandem repeat is directly related to the mechanical properties of spider silk, such as its strength, studies using recombinant proteins often focus exclusively on the core repeat domain but the NTD and CTD globular proteins are also important for the solubility of the protein and formation of hierarchical structures[19,20], affecting the properties of the produced fiber[19].

Cell division continued until day 4, after which the bacteria entered the stationary phase (Fig. 2a, b, Table 1). The specific growth rate was highest on days 0-2, at 0.56 ± 0.13 (Table 1). After the second day, the amount of inorganic C or N taken up into the cells was measured using total C (TC), inorganic C (IC), and total N (TN) analyses. There was a rough correlation between cell growth and the amount of C and N assimilated each day of culture (Fig. 2b, Table 1). This result is reasonable considering that amino acids are the main components of microorganisms and that the absorbed CO_2/HCO_3^- ions and N_2 gas are synthesized into amino acids[21,22]. The intracellular C:N molar ratio was roughly constant, ranging from 7.9 to 10.7 (Table 1), regardless of the specific growth rate (Table 1). The C:N ratio of marine bacteria has been reported to range from 5.0 to 8.3[23], suggesting that the cells in this culture system were prone to nitrogen deficiency stress.

The intracellular protein expression of MaSp2 was not detected by Coomassie blue staining but rather by Western blotting (Fig. 2c). MaSp2 expression per cell was highest on day 2 (Fig. 2c) of the logarithmic growth phase, but the culture was continued until the stationary phase to obtain more cells (Fig. 3a). After 8–10 days of incubation, TC, IC and TN analyses using the medium collected on the final day of each culture (Table 2) revealed that approximately 65.5 ± 27.9 mg/L and 5.4 ± 1.1 mg/L inorganic C and N were taken up into the cells (Table 2) and 1.6 ± 0.7 g wet cells were collected (Table 2).

Partial purification of the MaSp2 protein

After sonication, cell lysates were passed through a standard affinity Ni–nitrilotriacetic acid (NTA) column[24]. The N-terminus of the coding region of MaSp2 contains six consecutive histidine residues (Fig. 1c). The affinity of these residues for Ni-NTA was exploited to bind the MaSp2 protein to the column, which was then eluted by

Table 2 Analysis of cultures on the final day of cultivation used for MaSp2 purification by TOC or TN instruments, the amount of cells harvested, and the amount of MaSp2 collected.

	TOC	TC	IC	TN	Used C	Used N	Cells	TSP	TSP		MaSp2 in TSP	MaSp2	MaSp2	MaSp2
	(mg/L)	(mg/L)	(mg/L)	(mg/L)	(mg/L)	(mg/L)	(g, wet)	(μg/9 L)	(μg/L)	(μg/g wet cell)	(%)	(μg/9 L)	(μg/L)	(μg/g wet cell)
Culture	55.2 ± 28.0	373.4 ± 43.9	318.2 ± 31.6	10.8 ± 1.8										
Supernatant	30.5 ± 23.3	338.4 ± 41.1	307.9 ± 29.8	5.3 ± 0.7										
					65.5 ± 27.9	5.4 ± 1.1	1.6 ± 0.7	192.7 ± 125.2	21.4 ± 13.9	141.8 ± 128.6	12.1 ± 9.0	28.6 ± 34.1	3.2 ± 3.8	24.6 ± 34.7

Analysis of the culture medium or supernatant collected on the final day of incubation using the TOC/TN instrument, the values of C or N taken up into the cells, cell and MaSp2 yields in the culture and purification experiments. TSP: total soluble protein. Each value is the average ± SD of three independent experiments.

competition with imidazole. The MaSp2 protein band was detected in 10 μL of the Ni–NTA column eluate (approximately 1/50 of each elution fraction), indicating that the protein was concentrated (Fig. 3b). The three fractions with the highest MaSp2 elution were mixed and ultrafiltered, and the solvent was replaced with purified H_2O, resulting in the purification of MaSp2 (Fig. 3c). The total soluble protein (TSP) content resulted in 21.4 ± 13.9 μg/L (141.8 ± 128.6 μg/g wet weight cell), with the MaSp2 protein accounting for 12.1 ± 9.0% of the total soluble protein (TSP) content, resulting in 3.2 ± 3.8 μg/L (24.6 ± 34.7 μg/g wet weight cell) MaSp2 (Table 2 and Supplementary Fig. 1).

Discussion

From a sustainability perspective, we focused on the use of inorganic resources in bacterial cultivation, using the reduced inorganic sulfur compound $Na_2S_2O_3$ as the electron donor and gaseous CO_2/N_2 as the C/N source. Artificial seawater was used as the basal medium because the use of seawater is considered superior to the use of freshwater because it utilizes an unused resource. Under these sustainable and autotrophic conditions, a soluble form of the spider silk protein MaSp2, 3.2 ± 3.8 μg/L (24.6 ± 34.7 μg/g wet weight cell) (Table 2), was obtained from *R. sulfidophilum*. Previously, our research group cultured *R. sulfidophilum* carrying a plasmid encoding the gene for ampullate spidroin MaSp1 in seawater supplemented with 12 mM $NaHCO_3$ (equivalent to an IC of 143 mg/L) and N_2 gas, resulting in little cell growth that did not support further investigation[5]; under these experimental conditions, the yield of MaSp1 was 0.12 ± 0.10 mg/L, which was estimated to be derived from seed culture cells using organic N/C-rich MB medium. However, in the present study, *R. sulfidophilum* proliferated under similar autotrophic conditions when $Na_2S_2O_3$ was added as an electron donor. Thus, sufficient and balanced electron donors and N and C sources are still important even for the autotrophic bacterial production of exogenous proteins. On the other hand, in the same paper, the addition of 0.4 g/L yeast extract in seawater supplemented with 12 mM $NaHCO_3$ and N_2 gas promoted cell growth and increased MaSp1 protein yield to 3.9 ± 2.8 mg/L, and when MB medium containing abundant organic N/C sources were used, the MaSp1 yield was 52.3 ± 11.2 mg/L[5], which is comparable to that of heterologous protein expression systems in cyanobacteria[25]. These data indicate that under photoheterotrophic conditions, production of spider silk protein or possibly other heterologous proteins by *R. sulfidophilum* can increase from a few to tens of mg/L or mg/g cell levels. We speculate that such high levels of heterologous protein expression may be achievable even under autotrophic culture conditions if the efficiency of

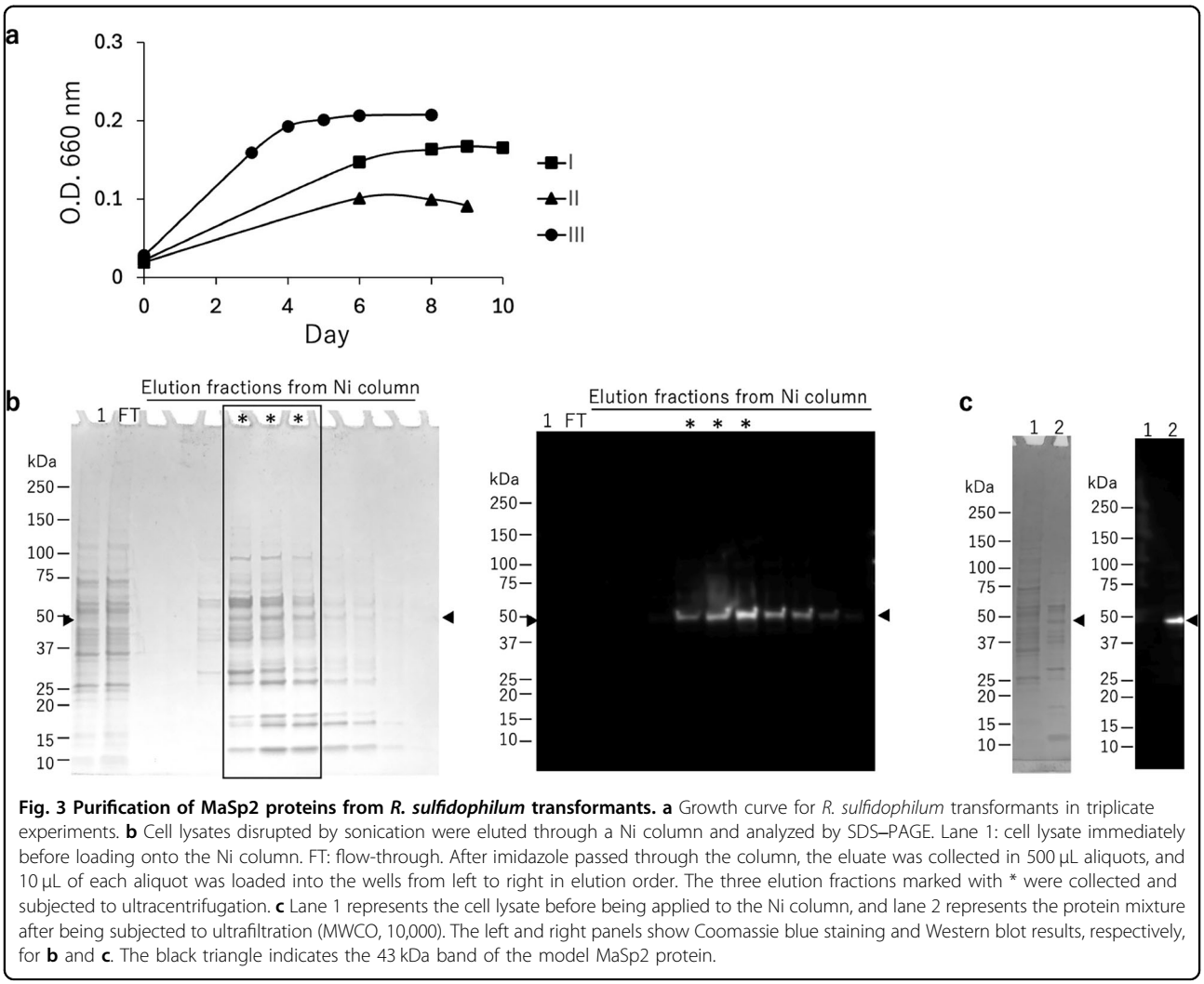

Fig. 3 Purification of MaSp2 proteins from *R. sulfidophilum* transformants. a Growth curve for *R. sulfidophilum* transformants in triplicate experiments. **b** Cell lysates disrupted by sonication were eluted through a Ni column and analyzed by SDS–PAGE. Lane 1: cell lysate immediately before loading onto the Ni column. FT: flow-through. After imidazole passed through the column, the eluate was collected in 500 µL aliquots, and 10 µL of each aliquot was loaded into the wells from left to right in elution order. The three elution fractions marked with * were collected and subjected to ultracentrifugation. **c** Lane 1 represents the cell lysate before being applied to the Ni column, and lane 2 represents the protein mixture after being subjected to ultrafiltration (MWCO, 10,000). The left and right panels show Coomassie blue staining and Western blot results, respectively, for **b** and **c**. The black triangle indicates the 43 kDa band of the model MaSp2 protein.

photosynthesis is improved and, consequently, the level of intracellular organic C is increased.

Data on the molar ratio of C and N incorporated into cells revealed that cells tend to be in an N-starved state when both CO_2 and N fixation were simultaneously induced (Table 1). The C:N ratio of marine bacteria has been reported to be in the range of 5.0 to 8.3[23], but in this study, the C:N ratio obtained was in the range of 7.9 to 10.7 (Table 1), suggesting that the current method only achieves the minimum N fixation required for the cells, and N fixation is the rate-limiting factor in this system. Because 16 mol of ATP is required to fix 1 mol of N_2, and ATP is also required for the subsequent amino acid synthesis, it is presumed that ATP is insufficient under the culture conditions. We speculate that restoring the intracellular C:N ratio to the standard levels (5.0 to 8.3) can be achieved by increasing the efficiency of photosynthesis, as mentioned above, to increase the amount of ATP available for N fixation and amino acid synthesis. When N-starved state is alleviated, an overall increase in amino acid synthesis is expected, along with increased production of MaSp2.

Compared with the growth of PNSB in common mineral medium[26,27], the bacterial growth in the medium used herein was slightly lower, even though the seawater in the present study was presumably supplemented with sufficient C, N, and electron sources. We recently reported that when photosynthesis and N fixation by nitrogenase were simultaneously performed in mineral medium containing vitamins and trace metal elements instead of seawater, the growth of *R. sulfidophilum* was suppressed compared with that of *R. sulfidophilum* in heterotrophic medium[22]. We therefore consider that the low growth rate is not due to a lack of vitamins and/or trace metal elements in the culture medium but rather to a lack of other components that properly regulate the assimilation of C and N. Alternatively, the necessary components may be present in

the seawater and the mineral medium above, but the concentrations of these components may need to be adjusted to efficiently induce both CO_2 and N fixation simultaneously. Phosphorus and iron are well-known rate-limiting factors for the growth of marine diazotrophs[28]; hence, they could be candidates for controlling photosynthetic activity and cellular growth. In the future, we will attempt to precisely adjust the concentrations of these potential components.

We speculate that manipulating the promoter region of the plasmid may also increase the production of spider silk proteins. The plasmid used in this study constitutively expresses a heterologous protein that is thought to be a constant source of stress for cells; controlling the timing of heterologous protein expression would reduce the stress, promote cell growth, and ultimately increase protein production.

As shown in Fig. 1c, the 6xMaSp2 repeat sequences of the MaSp2 protein are particularly enriched for certain amino acids, such as alanine, glutamine, and glycine, which would lead to a shortage of intracellular tRNAs corresponding to these amino acids. Introduction of genes encoding these tRNAs is also expected to lead to increased protein production. Besides, codon optimization and 5' untranslated region engineering may be promising options to increase spider silk production[15,29].

It is well known that under photoheterotrophic conditions, the metabolism of PNSB varies significantly depending on the intensity, wavelength, and duration of light irradiation[30,31]. Nitrogen fixation in PNSB also depends on light[32]. Because our study was conducted under photoautotrophic conditions, the role of photosynthesis is likely to be more important than in previous studies. Light irradiation conditions may be a factor that determines the amount of heterologous protein produced, and further investigation of light irradiation conditions is essential.

Alterations to the culture method may also increase cell numbers and heterologous protein production. In this study, we performed batch culture, in which all components were added before starting the culture. However, perfusion culture, in which old medium is removed and new medium is added periodically, may yield better results[33] because perfusion culture is expected to remove harmful waste products and add trace elements such as metals contained in seawater.

Like PNSB, cyanobacteria could fix CO_2 and N_2, and have been established early as tools for producing a wide range of useful substances[1]. Compared to cyanobacteria, PNSB has two major advantages as a host for recombinant proteins: first, it can utilize a wide range of substances as electron donors, and second, it does not require or produce oxygen during cultivation. While most cyanobacteria use H_2O as the sole electron source during photosynthesis, PNSB cannot use H_2O as an electron source but can utilize a variety of organic carbon compounds, including lignin, and reduced inorganic compounds as electron sources[34]. In particular, *R. sulfidophilum* can utilize sulfur compounds such as $Na_2S_2O_3$ and H_2S[11], it is expected that various waste materials from food factories, paper factories, and factories that emit sulfur-containing wastewater and exhaust gases can be used as electron sources to grow bacteria. Nitrogen fixation is highly sensitive to oxygen and can even be inhibited by environmental oxygen levels, but cyanobacteria require oxygen supply to grow and release oxygen during photosynthesis[35]. Although cyanobacteria have several sophisticated mechanisms to mitigate the inhibitory effect of oxygen on nitrogen fixation, the high concentrations of oxygen still inhibit nitrogen fixation[35]. PNSB neither requires nor produces oxygen, which may make them superior to cyanobacteria in simultaneous photosynthesis and nitrogen fixation induction.

The Cartagena Protocol on Biosafety is an international agreement that mandates the prevention of the spread of genetically modified microorganisms into the environment. Each country has its own national regulations based on the Cartagena Protocol. For example, in Japan, recombinant *R. sulfidophilum* must be grown in a controlled environment, and the risk of the bacteria spreading into the environment is considered low; cultivation must be carried out in a laboratory with specific equipment, and the culture medium must be inactivated (e.g., autoclaved) at the end of the experiment. However, if the scale of aquaculture expands to several hundred liters in the future, the risk of spreading to the environment is expected to increase. In case they do spread into the environment, it would be necessary to establish a method to prevent genetically modified organisms from multiplying there. Auxotrophic strategies that require nutrients that are rarely present in the environment should be introduced in this system in future[36].

This study demonstrated that under autotrophic conditions, both recombinant and exogenous proteins were reliably produced; however, the addition of high concentrations of electron donors and CO_2/N_2 gas was still insufficient to achieve sufficient protein production through photosynthesis and N fixation for industrial applications. To improve and enhance autotrophic productivity, some strategic modifications to culture conditions are needed to drive these metabolic processes efficiently. Since N fixation by nitrogenase appears to be rate-limiting and photosynthesis and N fixation are closely related[22,37], improvement in the N fixation pathway could increase photosynthesis via reduced sulfur compounds, resulting in increased biomass production from the photosynthetic process.

Methods

Growth conditions

R. sulfidophilum (ATCC35886/DSM1374) was obtained from the American Type Culture Collection (ATCC). *R. sulfidophilum* containing the plasmid was precultured with mineral medium M6 containing 20 mM $NaHCO_3$ as the inorganic C source, 7.6 mM $(NH_4)_2SO_4$ as the fixed N source, 8 mM $Na_2S_2O_3$ as the electron source, and 50 μg/mL kanamycin. The composition of the M6 medium per 1 L was as follows: K_2HPO_4, 0.78 g; KH_2PO_4, 0.75 g; $CaCl_2{\cdot}2H_2O$, 0.029 g; $MgSO_4{\cdot}7H_2O$, 0.247 g; $FeSO_4{\cdot}7H_2O$, 0.011 g; NaCl, 20 g; vitamin solution, 10 mL; and trace element mixture, 10 μL. The pH was adjusted to 7.0 with NaOH, and the medium was sterilized by autoclaving at 121 °C for 15 min. The composition of the vitamin mixture per 1 L was as follows: nicotinamide acid, 1.0 g; thiamine, 1.0 g; biotin, 50.0 mg; PABA, 0.5 g; vitamin B12, 10.0 mg; Ca pantothenate, 0.5 g; folic acid, 0.5 g; pyridoxine HCl, 0.5 g; and EDTA (3Na), 2.0 g. The composition of the trace element mixture per 1 L was as follows: $MnSO_4{\cdot}4H_2O$, 11.2 g; $ZnSO_4{\cdot}7H_2O$, 2.9 g; $Co(NO_3)_2{\cdot}6H_2O$, 2.9 g; $CuSO_4{\cdot}5H_2O$, 2.5 g; H_3BO_3, 3.1 g; $Na_2MoO_4{\cdot}2H_2O$, 2.4 g; and EDTA (3Na), 41.2 g. Precultured cells in the logarithmic growth phase in M6 medium were collected, and the cell pellets were washed and concentrated with artificial seawater (AIR WATER, Osaka, Japan). Cell density was assessed by measuring the absorbance of the culture medium at 660 nm using a spectrophotometer (AS ONE, Osaka, Japan). A total of 8.9 L of artificial seawater filtered through a 0.2 μm filter was placed in a 10 L capacity fermenter, which was equipped with a pH sensor to automatically inject acid or alkaline solutions to adjust the pH (Marubishi Bioengineering, Tokyo, Japan). Artificial seawater was used as the basal medium because the composition of seawater varies greatly depending on the season and collection location. CO_2 and N_2 gases were bubbled at rates of 0.15 L/min and 0.35 L/min, respectively, while stirring at 100 rpm for 2 h at 30 °C, and 1 N NaOH was added dropwise automatically. Approximately 250 mL of 1 N NaOH was used over the 2-h infusion, and the pH reached approximately 6.6. After the gas supply was stopped, 90 mL of 1 M $Na_2S_2O_3$ was added as an electron donor to a concentration of 9.7 mM, followed by the addition of 4.5 mL of 100 mg/mL kanamycin solution to a concentration of 49 μg/mL, and approximately 10 mL of the concentrated culture of *R. sulfidophilum* transformant was added to obtain an optical density of approximately 0.02 at 660 nm. The inorganic C concentration reached 340–420 mg/L 2 h after injection, as determined using a TOC instrument (Shimadzu, Kyoto, Japan). All parts of the fermenter exposed to the outside air were sealed, and the fermenter was kept at 30 °C under 730 nm far-red light with gentle stirring at 50 rpm. Light irradiation panels (CCS, Kyoto, Japan) were installed on both sides of the fermenter, and the incident light intensity on each was set to 20 W/m^2 and 170 μmol photons/m^2/s (380 nm to 780 nm). The λmax and full width at half maximum of the LED emission spectrum were 733 nm and 717–748 nm, respectively (Supplementary Fig. 2). The 730 nm wavelength was used because its effect on the growth of *R. sulfidophilum* has been studied. After 8–10 days, the culture was stopped. After the cells were collected by centrifugation, they were washed once with artificial seawater, and the pellet was stored at -80 °C until cell disruption and protein purification began. A total of 1.0–2.4 g of wet cells was collected in 3 experiments.

Plasmid construction and conjugation

A model MaSp2 coding region consisting of the N-terminal domain, six repeats of the tandem domain, and the C-terminus was transferred from the plasmid used in *Escherichia coli*[14] into a modified pBBR1MCS-2 broad-host-range vector[5]. The NTD and 6 repeats were derived from MaSp2 of *Trichonephila clavipes*, and the CTD was derived from MaSp2 of *Latrodectus hesperus*. The coding region was located downstream of the *trc* promoter, and there was a histidine tag at the N-terminus[5]. This plasmid was introduced into *R. sulfidophilum* via the RP4/RK2 conjugation system, as described previously[5]. Plasmid maps were generated using SnapGene® software (Dotmatics, MA, USA).

Analysis of MaSp2 protein expression levels

Based on the optical density values at 660 nm, equal numbers of cultured cells were collected by centrifugation. The cell pellet was suspended in Laemmli sample buffer containing 2-mercaptoethanol, heated at 100 °C for 5 min, and centrifuged at 13,000 rpm for 5 min. The supernatant was analyzed by SDS−PAGE on a 4–15% gradient gel (Bio-Rad, CA, USA), and protein bands were analyzed by Coomassie blue staining (Tokyo Chemical Industry, Tokyo, Japan) or semidry Western blotting using a PVDF membrane (Bio-Rad, CA, USA) and an anti-His-tag (6xHis-tag) antibody (MBL, Tokyo, Japan).

Protein purification

Fifty milliliters of purified water was added per 1 g of wet cells, and the suspended cells were subjected to sonication (TOMY SEIKO, Tokyo, Japan). The homogenate was centrifuged, and the supernatant was collected. The supernatant was adjusted to 20 mM sodium phosphate, 500 mM NaCl, and 20 mM imidazole and run over a 1 mL histidine-tagged column (HisTrap FF, Cytiva, Tokyo, Japan). The bound proteins were eluted via isocratic elution with 500 mM imidazole. Approximately 1.5 mL of the eluate was subjected to ultrafiltration (Cytiva, Tokyo, Japan, molecular weight cutoff of 10,000), replaced with H_2O by adding 5 mL of H_2O three times, and concentrated to a final volume of 300–500 μL. The protein concentration was determined using the BCA

protein assay (Thermo Fisher Scientific, MA, USA). Soluble protein was subjected to SDS–PAGE on a 4–15% gradient gel (Bio-Rad, CA, USA) and detected by Coomassie blue staining (Tokyo Chemical Industry, Tokyo, Japan) or semidry Western blotting using a PVDF membrane (Bio-Rad, CA, USA) and an anti-His-tag (6xHis-tag) antibody (MBL, Tokyo, Japan). The amount of MaSp2 in TSP was calculated using Coomassie-stained gels by image analysis software (ATTO, Tokyo, Japan). The validity of this calculation method was confirmed by comparing the obtained protein solution with a purified His-tagged standard protein solution using Coomassie staining and Western blotting (Supplementary Fig. 1).

Analysis of the amount of carbon and nitrogen taken up into cells

The culture mixture and the supernatant obtained by centrifugation (9000 g, 10 min, 4 °C) were subjected to total organic carbon (TOC) and total nitrogen (TN) analyses (Shimadzu, Kyoto, Japan), respectively, to determine the total carbon (TC, mg/L), inorganic carbon (IC, mg/L), and total N (TN, mg/L) in each sample. The total organic carbon (TOC, mg/L) content was calculated by subtracting the IC value from the TC value. The amounts of carbon and nitrogen taken up into the cells were calculated using the following formulas:

Carbon taken up into cells (mg/L) = TC (mg/L) of the culture − IC (mg/L) of the supernatant.

Nitrogen taken up into cells (mg/L) = TN (mg/L) of the culture − TN (mg/L) of the supernatant.

TOC analyzers use heat and a platinum catalyst to decompose TC into CO_2, and measure the amount of CO_2 gas as the peak area in the detector[38,39]. By calculating the ratio between this value and the area value of the TC standard solution, the TC concentration in the sample can be calculated. The amount of IC is determined by acid degradation of the sample (pH below 3), which releases CO_2 from all carbonates in the sample, which is then measured in the same way as TC[38,39]. The amount of TN is measured by heating the sample to 720 °C, which causes the TN in the sample to decompose into nitric oxide, which is then analyzed in the gas analyzer and detected as a peak area[39]. However, this method cannot measure N_2 gas because N_2 gas in the sample is not converted to nitric oxide even when heated to 720°C. Therefore, fixed nitrogen was measured indirectly by subtracting the TN value of the supernatant from the TN value of the culture medium.

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Author contributions
K.N. conceived the original research idea. M.S. and K.N. designed the experiments. M.S. conducted the experiments. M.S. and K.N. analyzed the results. M.S. wrote the manuscript. All the authors reviewed the manuscript.

Data availability
The raw data were generated at Kyoto University and the RIKEN Center for Sustainable Resource Science. Derived data supporting the findings of this study are available from the corresponding author (K.N.) upon request.

Competing interests
Keiji Numata is the CTO of Symbiobe Inc., a start-up company affiliated with Kyoto University.

Ethics approval and consent to participate
All genetic recombination experiments were performed in accordance with the guidelines and regulations of Kyoto University and were approved by the Kyoto University Recombinant DNA Experiment Safety Committee (Approval no. 250049).

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