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Biofixation of high-concentration carbon dioxide using a deep-sea bacterium: *Sulfurovum lithotrophicum* 42BKT^T

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Concerns about global warming have dramatically increased interest in the development of a strategy for the sustainable and economical fixation of carbon dioxide (CO_2). Achieving treatment of high-concentration CO_2 (e.g., higher than 1 atm CO_2) under a minute thermal energy penalty (e.g., at room temperature) is a significant goal for efficient fixation. Herein, we report the great potential of *Sulfurovum lithotrophicum* 42BKT^T, a deep-sea sulfur-oxidizing bacterium, for the biofixation of CO_2 at a pressure as high as 2 atm CO_2, mixed with 8 atm N_2, at 29°C. *Sulfurovum lithotrophicum* 42BKT^T exhibited fast specific fixation rate of approximately 0.42 g CO_2 g cell^{-1} hr^{-1}. Moreover, 37% of the total CO_2 fixation was present in the form of five dominant metabolites, especially glutamate and pyroglutamate. This work constitutes an efficient biofixation strategy of high-concentration CO_2 by using a deep-sea chemolithoautotrophic bacterium.

Introduction

Global warming caused by anthropogenic greenhouse gas (GHG) emissions has become one of the most important issues in the world, as GHG is believed to be responsible for the current global climate change.[1] Carbon dioxide (CO_2) derived from fossil fuel combustion accounts for up to 68% of the total GHG emissions; however, CO_2 is also essential to photosynthesis and other photoautotrophic processes as a part of the Earth's carbon cycle.[2] Therefore, much research and development has been directed toward reducing the level of CO_2 in the atmosphere. As a result of these efforts, various strategies have been suggested for CO_2 reduction. These strategies mainly fall into one of the two following categories: 1) carbon capture and underground storage (CCS) and 2) biological or chemical conversion to organic matter.[2]

The capture of CO_2 requires the separation of CO_2 from its industrial and energy-related sources. Captured CO_2 can then be compressed, transported through pipelines, and stored in geological features at great depth. For example, captured CO_2 can be stored in depleted oil/gas reservoirs, unmineable coal beds, and saline aquifers; considering all the sedimentary basins in the world, the capacity of geological storage is potentially huge.[3] In the present state of technology, CCS is an expensive solution that requires great energy consumption.[3] In addition, due to the great distances often separating the sites of CO_2 emission and storage, very long pipelines are often needed to transport the highly compressed CO_2. Moreover, sometimes these pipelines pass through other provinces or countries not involved with either CO_2 emission or CO_2 storage. Therefore, the sustainability, safety, and public acceptance of geological storage should be confirmed prior to its implementation at a particular site.[4]

Biofixation of CO_2 is a natural process carried out by all terrestrial plants as part of photosynthesis, as well as through the photosynthetic and assimilative activities of a tremendous number of microorganisms. Biofixation of CO_2 is believed to be both economically and environmentally advantageous, since it often results in the coproduction of industrially valuable biomasses.[2, 5] Microalgae, for example, have garnered much attention because they can convert CO_2 into biomasses via photosynthesis at much higher rates than terrestrial plants.[6] The potential applications of microalgae range from the production of fine chemicals, including pigments, polyunsaturated fatty acids,[7] and biodiesel;[8] microalgae can also be used to remove CO_2 from flue gases.[9] However the productive culture of microalgae requires a regulated supply of homogeneously distributed light, whose intensity is carefully controlled.[10] For this purpose, tubular photobioreactors, flat panel photobioreactors, or column photobioreactors with external light supplies, large surface areas, short internal light paths, and small dark zones have been proposed as potential photobioreactors for microalgae.[11-13] Optimum growth of microalgae has been reported at low CO_2 concentrations such as 5-20% under ambient pressure (i.e., a partial CO_2 pressure of 0.05-0.2 atm); some species of microalgae even tolerate CO_2 concentrations as high as 50-100% (i.e., a partial CO_2 pressure of 0.5-1.0 atm).[14-17] Hence, large areas/spaces and adequate conversion times are likely going to be required in order to use

photosynthetic organisms, such as microalgae, for meaningful removal of CO_2 from the massive amount of emission gases.

The most common use of CO_2 is as a chemical reagent for the production of urea, which is used in around 90 percent of the world's fertilizers[18] Recently, the catalytic conversion of CO_2 to alkynyl carboxylic acid and to cyclic carbonate was reported.[19] Another study described a novel reaction in which CO_2 was used in place of carbon monoxide for carbonylation reactions.[20] Such chemical conversion methods have the advantage of high reaction rates at high CO_2 concentrations. However, most methods of CO_2 chemical conversion require expensive catalysts, high temperature, or high pressure.[21] Thus, the overall net emission of CO_2 must be taken into consideration, meaning that conversion efficiencies exceeding those attainable with the present technologies are urgently needed.

Chemolithoautotrophs grow on the energy released from the oxidation of inorganic compounds (e.g. S^{2-}, $S_2O_3^{2-}$, NH_3, H_2, etc.) and utilize inorganic carbons (e.g. CO_2 and HCO_3^-) as sources of carbon.[22] Hence the chemolithotrophs need chemical energy for the conversion of CO_2 while the microalgae and the chemical conversion methods need the homogeneously distributed/intensity-controlled light energy and the thermal energy, respectively. If a chemolithoautotrophic bacterium is capable of CO_2 assimilation at low temperature and high CO_2 concentrations (e.g., 25°C and over 1 atm CO_2), it would achieve CO_2 fixation under the less thermal energy penalty than chemical conversion methods. It would also make CO_2 fixation feasible in smaller spaces than microalgae and bypass concerns related to the limited range in the CO_2 concentrations.

In this study, *Sulfurovum lithotrophicum* 42BKT^T (JCM 12117^T), a deep-sea sulfur-oxidizing bacterium, was used as a candidate of chemolithoautotrophs for the biofixation of CO_2 at partial CO_2 pressures up to 2 atm. This bacterium has been reported to successfully grow at a partial CO_2 pressure of 0.3 atm (in a total pressure of 1.5 atm), using thiosulfate and nitrate as an electron donor and acceptor, respectively.[23] We predicted that the cell membrane of this deep-sea living bacterium would enable it to withstand the high CO_2 concentrations (high concentrations of CO_2(aq), H_2CO_3(aq), HCO_3^-, and CO_3^{2-} generated under these elevated CO_2 pressures). To the best of our knowledge, this study is the first to investigate whether a deep-sea living chemolithoautotroph can perform biofixation of CO_2 at a partial CO_2 pressure greater than 1 atm. The rate of CO_2 fixation at CO_2 pressure of 2 atm was compared with that at the lower CO_2 pressure (e.g., 0.3 atm) to confirm the feasibility of CO_2 biofixation by this bacterium in a wide range of CO_2 pressure. An inorganic carbon source was supplied to seawater in the form of either a CO_2/N_2 gas mixture, or sodium bicarbonate salt ($NaHCO_3$). The CO_2/N_2 gas mixture (pressure ratio, 2:8) was used as a model for flue gases emitted from power plants using fossil fuels, with the caveat that the composition of actual flue gases has been shown to vary somewhat depending on the type of fuel used, the combustion system, and the degree of combustion (e.g., 5% ≤ CO_2 ≤ 25%, 70% ≤ N_2 ≤ 80%[24]).

Results and discussion

The results of culture experiments in which the CO_2/N_2 gas mixture was supplied as the carbon source are shown in Figure 1 and Table 1. *Sulfurovum lithotrophicum* 42BKT^T successfully performed CO_2 fixation under a partial CO_2 pressure of 2 atm (Figure 1), which is a relatively high CO_2 pressure in the context of biofixation. Once CO_2 is dissolved in seawater, CO_2 is present in various forms such as CO_2(aq), H_2CO_3(aq), HCO_3^-, and CO_3^{2-}. Hence, the amount of CO_2 fixation by *Sulfurovum lithotrophicum* 42BKT^T was determined by carbon analysis of the dry cells and the extracellular metabolites.

The specific rate of CO_2 fixation, q_C, was similar regardless of the initial partial pressure of CO_2 ($p^0_{CO_2}$) and the initial total pressure (P^0_{total}): 0.44, 0.40, 0.42 and 0.41 g CO_2 g cell^{-1} hr^{-1} at $p^0_{CO_2}$ values of 0.3, 0.6, 1.4 and 2.0 atm (i.e., P^0_{total}= 1.5, 3.0, 7.0 and 10.0 atm), respectively. The growth rate of the dry cell mass was also similar, regardless of $p^0_{CO_2}$, even though the growth rate of the cell number density abruptly decreased with the increase of $p^0_{CO_2}$ from 0.6 to 2.0 atm (i.e., with the increase of P^0_{total} from 3.0 to 10.0 atm). Note that the latter growth rate is plotted on a logarithmic scale in Figure 1. This result was in agreement with the abrupt increase in cell size observed with the increase in pressure (Figure 2).

Five metabolites were identified as the dominant species through metabolomic analysis: succinate, lactate, aspartate, pyroglutamate, and glutamate (Table 1). Of particular note, the levels of pyroglutamate and glutamate were much higher than those of succinate, lactate, and aspartate in both the exterior and the interior of the cells. The extracellular ($q_{P,ext}$) levels of the five metabolites, especially pyroglutamate and glutamate, increased most rapidly at a $p^0_{CO_2}$ of 2.0 atm (P^0_{total}= 10 atm), even though these extracellular levels were smaller than the corresponding intracellular ($q_{P,int}$) levels. The intracellular levels of pyroglutamate and glutamate were lowest at the highest pressure (i.e., $p^0_{CO_2}$=2.0 atm and P^0_{total}= 10 atm). These results can probably be at least partially attributed to the secretion of pyroglutamate and glutamate.

Glutamate has been reported to be converted to pyroglutamate via enzymatic dehydration.[25] Assuming that pyroglutamate was formed from glutamate, glutamate and pyroglutamate were considered as a single species and designated as total glutamate in this study. The specific rates of the increases in the extracellular ($q_{P,ext}$) and intracellular ($q_{P,int}$) levels of total glutamate were recalculated, and are shown in Table 1. In chemolithoautotrophs, glutamate is known to be synthesized from 2-oxoglutarate,[26] which is not only one of the key starting points in the metabolic network of chemolithoautotrophs, but is also one of the major intermediates in the reductive tricarboxylic acid (reductive

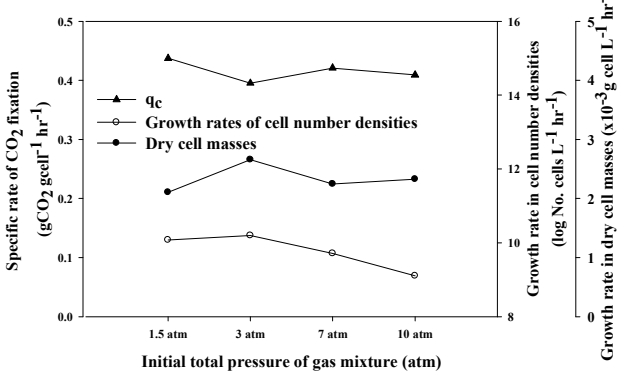

Figure 1 Results of the 24 h culture experiments (29°C) in which the carbon source was a CO_2/N_2 gas mixture ($p^0_{CO_2}$: $p^0_{N_2}$= 2:8).

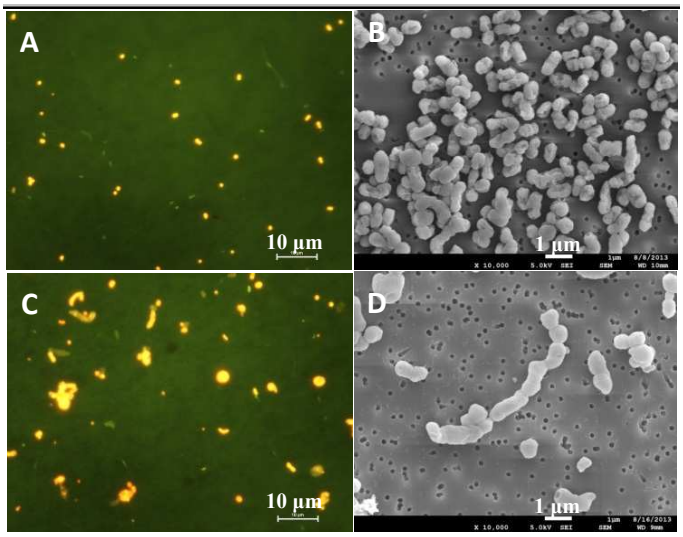

Figure 2 Results of the 24 h culture experiments (29°C) in which the carbon source was a CO_2/N_2 gas mixture ($p^0_{CO_2}$: $p^0_{N_2}$= 2:8).

TCA) cycle. This cycle was reported to be the pathway of CO_2 assimilation in *Sulfurovum lithotrophicum* 42BKT^T.[23] Succinate is one of the intermediates in the reductive TCA cycle. Lactate has been known to be synthesized from pyruvate via dehydrogenation in anaerobic metabolism. Pyruvate might be synthesized in *Sulfurovum lithotrophicum* 42BKT^T from acetyl-CoA, a product of the reductive TCA cycle. Aspartate has been known to be synthesized from oxaloacetate, which is also one of the major intermediates in the reductive TCA cycle. It was proposed that all products of autotrophic metabolism are funneled into anabolic pathways, with all inputs completely utilized;[26] therefore, no waste products are generated. In *Sulfurovum lithotrophicum* 42BKT^T, however, the accumulation and secretion of glutamate (and pyroglutamate) have been demonstrated, even though the secreted amount was quite small compared to the amount of intracellular glutamate. However, *Sulfurovum lithotrophicum* 42BKT^T did not appear to respond to the increased levels of CO_2 by accumulating intracellular total glutamate, since the intracellular level of total glutamate was lower at higher CO_2 pressures (Table 1).

As an alternative carbon source to CO_2, $NaHCO_3$ was added to the culture medium and the headspace was filled with N_2 gas. The molar amount of converted bicarbonate was then determined by analyzing the dominant extracellular metabolites and the cellular carbons; this amount was then multiplied by the molecular weight of CO_2 to compare these results to the results obtained when the CO_2/N_2 gas mixture was supplied as the carbon source (Figure 1).

The specific rate of CO_2 fixation (q_C) was similar regardless of either the amount of $NaHCO_3$ added, or the gas (N_2) pressure: 0.48, 0.40, 0.40 and 0.40 g CO_2 g cell^{-1} hr^{-1} at 0.41 g $NaHCO_3$ L^{-1} (P^0_{total}= 1.5 atm), 1.31 g $NaHCO_3$ L^{-1} (P^0_{total} = 3 atm), 4.62 g $NaHCO_3$ L^{-1} (P^0_{total} = 7 atm) and 8.59 g $NaHCO_3$ L^{-1} (P^0_{total}= 10 atm), respectively (see Figure 3). Similar to when gaseous carbon was supplied, the growth rate of cell number density abruptly decreased as the amount of $NaHCO_3$ added (and the N_2 pressure) increased from 1.31 to 8.59 g $NaHCO_3$ L^{-1} (and from 3 to 10 atm), whereas the growth rates of dry cell masses were similar; the largest cells were found at the highest pressure and dose of $NaHCO_3$ (Figure S3). Moreover, $q_{P,ext}$ of pyroglutamate and glutamate abruptly increased at the highest pressure and dose of $NaHCO_3$ (Table 2). As with the cultures supplied with gaseous carbon, the lowest intracellular levels of pyroglutamate and glutamate were observed at the highest pressure. This result might be partially due to secretion of these metabolites (see $q_{P,int}$ of pyroglutamate and glutamate in Table 2).

An increase in cell size (i.e., the emergence of lengthened cells) was observed at the highest $p^0_{CO_2}$ and P^0_{total} when the CO_2/N_2 gas mixture was supplied as the carbon source (Figure 2C and 2D); moreover, a similar increase was seen at the highest dose of $NaHCO_3$ and the highest P^0_{total} when $NaHCO_3$ was supplied as the carbon source (Supplementary Information Figures S3C and S3F). To determine whether the CO_2 concentration in the culture medium or the pressure in the head space resulted in this cell lengthening, cell cultivation was performed at a constant $NaHCO_3$ concentration and under different pressures. The initial concentration of $NaHCO_3$ was

Table 1 Specific rates of the increases in the extracellular ($q_{P,ext}$) and intracellular ($q_{P,int}$) levels of five dominant metabolites during the 24 h culture at 29°C in which the carbon source was a CO_2/N_2 gas mixture.[a]

	$q_{P,ext}$[b]				$q_{P,int}$[b]			
	1.5 atm	3 atm	7 atm	10 atm	1.5 atm	3 atm	7 atm	10 atm
Succinate	0.05	0.04	0.05	0.05	0.16	0.08	0.12	0.16
Lactate	0.0	0.01	0.02	0.03	0.69	0.49	0.44	0.85
Aspartate	0.0	0.0	0.05	0.33	3.28	2.28	1.31	1.93
Pyroglutamate	0.0	0.0	0.38	0.93	22.5	20.1	13.5	8.90
Glutamate	0.41	0.42	0.54	1.50	68.7	64.4	56.3	55.0
Total glutamate[c]	0.41	0.42	0.97	2.56	94.4	87.3	71.7	65.1

[a] $q_{P,ext}$ and $q_{P,int}$ were calculated from equations (8) and (9) using the data in Table S1. μ was assumed to be 0.234 hr^{-1}. The pressures given in this table (i.e., 1.5, 3.0, 7.0 and 10 atm) are the total pressures, which were initially controlled with a CO_2/N_2 gas mixture at a 2:8 ratio (i.e., $p^0_{CO_2}$: $p^0_{N_2}$= 2:8).

[b] Quantities given are in units of mg metabolite g cell^{-1} hr^{-1}.

[c] Assuming the formation of pyroglutamate from glutamate, glutamate and pyroglutamate were considered as a single species, designated as total glutamate in this study. The values of $q_{P,ext}$ and $q_{P,int}$ of total glutamate were calculated as follows:

$$q_{P,ext}(\text{total glutamate}) = q_{P,ext}(\text{pyroglutamate}) \times \frac{147}{129} + q_{P,ext}(\text{glutamate})$$

$$q_{P,int}(\text{total glutamate}) = q_{P,int}(\text{pyroglutamate}) \times \frac{147}{129} + q_{P,int}(\text{glutamate})$$

where 147 and 129 are the molecular weights of glutamate and pyroglutamate, respectively.

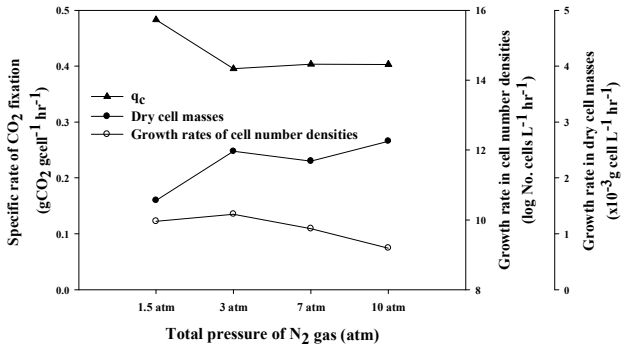

Figure 3 Results of the 24 h culture experiments (29°C) in which the carbon source was $NaHCO_3$. The amounts of converted bicarbonate were determined by analyzing the product carbons, expressed in units of g CO_2 for comparison with Figure 1

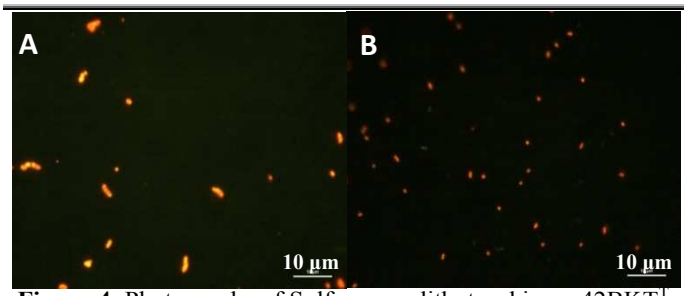

Figure 4. Photographs of Sulfurovum lithotrophicum 42BKT[T] cells in a consecutive culture, in which the inorganic carbon source was supplied in the form of a CO_2/N_2 gas mixture, but the initial partial pressure of CO_2 ($p^0_{CO_2}$) was reduced from 2.0 (A) to 0.3 (B) atm. After the first 24 hr culture under a $p^0_{CO_2}$ of 2.0 atm, cells (A) were harvested and transferred to a reactor containing fresh medium. The second 24 hr culture (B) was under a $p^0_{CO_2}$ of 0.3 atm

1.31 g L^{-1}. The head space was filled with nitrogen gas, and the total (nitrogen) pressure (i.e., $P^0_{total}(=p^0_{N_2})$) was varied from 3.0 to 30.0 atm. Lengthened cells were not observed at any pressure conditions, which indicated that the cell lengthening was caused not by the pressure in the head space of reactor, but by the increased CO_2 concentration in the culture medium (Figure S4). When the cells grown at the highest CO_2 concentration (e.g., a $p^0_{CO_2}$ value of 2.0 atm and a $p^0_{N_2}$ value of 8.0 atm) were transferred to medium with a lower CO_2 concentration (e.g., a $p^0_{CO_2}$ value of 0.3 atm and a $p^0_{N_2}$ value of 1.2 atm), cell growth continued; moreover, all of the cells had the same morphology and no lengthened cells were observed (Figure 4). Hence, we hypothesize that the cell lengthening observed at the highest CO_2 concentration is a reversible response to the stress of the high CO_2 level.

Thiosulfate is the electron donor and energy source for *Sulfurovum lithotrophicum* 42BKT[T]. Since its initial concentration was the same in all experiments (i.e., 1.5 g of $Na_2S_2O_3{\cdot}5H_2O$ per 1 l of MJ-N synthetic seawater), a specific growth rate (μ) of 0.234 hr^{-1}, which was determined at a $p^0_{CO_2}$ of 0.6 atm and a $p^0_{N_2}$ of 2.4 atm, was applied for all CO_2 fixation experiments, regardless of CO_2 pressures and bicarbonate concentrations. A μ value of 0.234 hr^{-1} corresponds to a doubling time (t_d) of approximately 3 hr. The specific growth rate of microalgae is generally known to be in the range of 0.0007 to 0.09 hr^{-1}, which corresponds to doubling times ranging from 8 hr to 40 days.[27-29] When 5% CO_2 was applicable, the CO_2 fixation rate of microalgae was reported to be in the range of 0.0008 to 0.0019 g CO_2 L^{-1} hr^{-1}.[27-29] As the density of micoalgal cells in the CO_2 fixation studies was about 1 g cell L^{-1}, the specific rate of CO_2 fixation would be in the range of 0.0008 to 0.0019 g CO_2 g $cell^{-1}$ hr^{-1}. Hence, *Sulfurovum lithotrophicum* 42BKT[T] is capable of assimilating CO_2 at a high rate even when the CO_2 concentration is high.

A coefficient for metabolite formation upon the increase in the cell mass, $Y_{P/X}$ (mg metabolite g $cell^{-1}$; see Eq. (6) in Methods), was used to calculate the $q_{P,ext}$ and $q_{P,int}$ values (the specific rate of metabolite formation in the exterior and interior of the cells; mg metabolite g $cell^{-1}$ hr^{-1}) of the dominant metabolites. The intracellular and extracellular values of $Y_{P/X}$ for the five dominant metabolites are shown in Tables S1 and

Table 2 Specific rates of the increases in the extracellular ($q_{P,ext}$) and intracellular ($q_{P,int}$) levels of five dominant metabolites during the 24 h culture at 29°C, in which the carbon source was $NaHCO_3$.[a]

	qP,ext[b]				qP,int[b]			
	1.5 atm 0.41 g L^{-1}	3 atm 1.31 g L^{-1}	7 atm 4.62 g L^{-1}	10 atm 8.59 g L^{-1}	1.5 atm 0.41 g L^{-1}	3 atm 1.31 g L^{-1}	7 atm 4.62 g L^{-1}	10 atm 8.59 g L^{-1}
Succinate	0.10	0.08	0.03	0.17	0.17	0.07	0.12	0.52
Lactate	0.0	0.02	0.00	0.0	0.81	0.33	1.14	1.62
Aspartate	0.0	0.0	0.01	0.24	5.32	3.20	2.49	4.61
Pyroglutamate	0.0	0.0	0.29	0.71	26.5	19.7	12.6	9.61
Glutamate	0.78	0.56	0.44	1.39	75.4	66.7	64.5	64.9
Total glutamatec	0.78	0.56	0.76	2.19	105.6	89.1	78.9	75.9

[a] $q_{P,ext}$ and $q_{P,int}$ were calculated from equations (8) and (9) using the data in Table S2. μ was assumed to be 0.234 hr^{-1}. The pressures given in this table (i.e., 1.5, 3.0, 7.0 and 10 atm) are the total pressures, which were controlled using N_2 gas only. The initial concentration of $NaHCO_3$ is given below the total pressure.

[b] Quantities given are in units of mg metabolite g $cell^{-1}$ hr^{-1}.

[c] Assuming the formation of pyroglutamate from glutamate, glutamate and pyroglutamate were considered as a single species, designated as total glutamate in this study. The values of $q_{P,ext}$ and $q_{P,int}$ of total glutamate were calculated as follows:

$$q_{P,ext}(\text{total glumate}) = q_{P,ext}(\text{pyroglutamate}) \times \frac{147}{129} + q_{P,ext}(\text{glutamate})$$

$$q_{P,int}(\text{total glutamate}) = q_{P,int}(\text{pyroglutamate}) \times \frac{147}{129} + q_{P,int}(\text{glutamate})$$

where 147 and 129 are the molecular weights of glutamate and pyroglutamate, respectively.

Table 3 Elemental composition and empirical chemical formula of *Sulfurovum lithotrophicum* 42BKT[T].

Composition (% by wt)[a]					Empirical chemical formula[b]
C	H	N	O	S	
48.66±0.48	7.22±0.07	13.32±0.17	27.50±1.80	1.10±0.00	$CH_{1.78}N_{0.23}O_{0.42}$

[a] Expressed in weight percentage. The average values and the standard deviations are given.
[b] Since the composition of sulfur was negligibly small, S was not included in the empirical chemical formula of *Sulfurovum lithotrophicum* 42BKT[T].

S2 (Supplementary Information). The extracellular amounts of the metabolites were much smaller than the intracellular amounts. When the CO_2/N_2 gas mixture was supplied as the carbon source (Table S1), the sums of the intracellular and extracellular $Y_{P/X}$ values were 410, 375, 311 and 298 mg metabolite g cell^{-1} for cultures under P^0_{total} of 1.5, 3.0, 7.0 and 10.0 atm, respectively. When $NaHCO_3$ was added as the carbon source (Table S2), these values were 467, 387, 349 and 359 mg metabolite g cell^{-1} for cultures under $P^0_{total}(= p^0_{N_2})$ of 1.5, 3.0, 7.0 and 10.0 atm, respectively. The average value of the sum of the intracellular and extracellular $Y_{P/X}$ values was 370 mg metabolite g cell^{-1}, with a standard deviation of 51 mg metabolite g cell^{-1}. Approximately 37% of the dry cell mass was these five metabolites. Assuming that the compositions of the cell dry masses and the five dominant metabolites were the same, approximately 37% of the assimilated CO_2 was present in the form of these five metabolites, especially glutamate and pyroglutamate.

The values of $Y_{X/S}$ (the yield coefficient of the carbon source; see Eq. (3) in Methods) are shown in Tables S1 and S2 (Supplementary Information). The fractions of carbon in the dry cell masses, which were used to calculate $Y_{X/S}$, were estimated from an elemental analysis of the dry cell masses and are shown in Table S3 (Supplementary Information). The elemental composition and empirical chemical formula of *Sulfurovum lithotrophicum* 42BKT[T] are shown in Table 3. From the analysis of thiosulfate and nitrate ions, *Sulfurovum lithotrophicum* 42BKT[T] was found to require 4.65±0.13 mmol of thiosulfate ($S_2O_3^{2-}$) and 7.53±0.18 mmol of nitrate (NO_3^-) for the fixation of 1 mmol of CO_2. The following material balance equation can therefore be used to describe the process of CO_2 fixation by *Sulfurovum lithotrophicum* 42BKT[T]:

$$CO_2 + 0.23NH_4^+ + 4.65S_2O_3^{2-} + 7.53NO_3^- \rightarrow CH_{1.78}N_{0.23}O_{0.42} + 9.3SO_4^{2-} + 3.77N_2$$

Hence, the oxidation of 1 mole of thiosulfate produced on average 5.3 grams of *Sulfurovum lithotrophicum* 42BKT[T]. This value is within the previously reported range of 5.1 to 11.4 g dry cell mass per mole of thiosulfate for chemolithoautotrophic bacteria utilizing thiosulfate and nitrate as the energy source and the electron acceptor, respectively.[30]

Overall, the specific biofixation rate and the cell growth rate were nearly the same, regardless of the CO_2 pressures and bicarbonate concentrations. Thus, we conclude that an increased supply of the inorganic carbon source (either CO_2 gas or $NaHCO_3$) does not decelerate the biofixation of CO_2 by *Sulfurovum lithotrophicum* 42BKT[T], and that CO_2 pressures even exceeding 1 atm can likely be biologically treated. The results of this study also suggest the feasibility of CO_2 biofixation in a wide pressure range of CO_2–containing gas mixtures (i.e., low pressure in an on-site reactor and high pressure in a reactor where compressed gas is supplied by pipeline transport). Moreover, the successful conversion at 29°C implies a minute thermal energy penalty for the biofixation of CO_2.

If a process for CO_2 biofixation by a deep-sea chemolithoautotrophic bacterium such as *Sulfurovum lithotrophicum* 42BKT[T] were developed and optimized through further studies, it would allow the reduction of CO_2 by a pressure bioreactor, built near the source of CO_2/N_2 emissions. And the successful biofixation of CO_2 when it was mixed with another gas (N_2) implies the reduced need of separation processes for CO_2 capture. Since this biofixation process would reduce the need for the capture and storage of CO_2, the expense of CO_2 separation and the high pressure transport for storage would also be reduced in CCS. Furthermore, if an emission site does not have enough available space for a sufficiently large bioreactor, the flue gas must be compressed and transported through pipelines to the bioreactor site. Then, the compressed CO_2 mixture could be directly treated without depressurization prior to its introduction into the bioreactor. Since the treatment could be in-situ (i.e., at the emission site) or ex-situ at a nearby site, the public may accept this treatment without any significant misgivings

Materials and methods

MJ-N synthetic seawater

MJ-N synthetic seawater was used to make the culture medium. The composition of MJ-N synthetic seawater is (per liter): 30.0 g NaCl, 0.14 g K_2HPO_4, 0.14 g $CaCl_2{\cdot}H_2O$, 3.4 g $MgSO_4{\cdot}7\ H_2O$, 4.18 g $MgCl_2{\cdot}6\ H_2O$, 0.33 g KCl, 0.5×10^{-3} g $NiCl_2{\cdot}6\ H_2O$, 0.5×10^{-3} g $NaSeO_3{\cdot}5\ H_2O$, 0.01 g $Fe(NH_4)_2(SO_4)_2{\cdot}6\ H_2O$, and 10 mL trace mineral solution. The trace mineral solution contains (per liter): 1.5 g nitrilotriacetic acid, 3.0 g $MgSO_4{\cdot}7H_2O$, 0.5 g $MnSO_4{\cdot}xH_2O$, 1.0 g NaCl, 0.1 g $FeSO_4{\cdot}7H_2O$, 0.1 g $CoSO_4{\cdot}7H_2O$, 0.1 g $CaCl_2{\cdot}2H_2O$, 0.1 g $ZnSO_4{\cdot}7H_2O$, 0.01 g $CuSO_4{\cdot}5H_2O$, 0.01 g AlK(SO4)2 0.01 g H_3BO_3, and 0.01 g $Na_2MoO_4{\cdot}2H_2O$.[23]

MJ-N basal medium

MJ-N basal medium was used for the preculture of *Sulfurovum lithotrophicum* 42BKT[T], and was prepared by adding 0.25 g of NH_4Cl, 1.5 g of $NaHCO_3$, 1.5 g of $Na_2S_2O_3{\cdot}5H_2O$, 2 g of $NaNO_3$, and 1 mL of trace vitamin solution to 1 L of MJ-N synthetic seawater.[23] The trace vitamin solution contains (per liter): 2.0 mg biotin, 2.0 mg folic acid, 10.0 mg pyridoxine·HCl, 5.0 mg thiamine·HCl, 5.0 mg riboflavin, 5.0 mg nicotinic acid, 5.0 mg calcium pantothenate, 0.1 mg vitamin B_{12}, 5.0 mg *p*-aminobenzoic acid, and 5.0 mg lipoic acid.

Modified MJ medium

The composition of MJ basal medium was modified to include 60 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), and to exclude $NaHCO_3$. The resulting medium contained 0.25 g of NH_4Cl, 1.5 g of $Na_2S_2O_3 \cdot 5H_2O$, 2 g of $NaNO_3$, 60 mmol of PIPES, and 1 mL of trace vitamin solution per 1 L of MJ-N synthetic seawater, and is referred to hereafter as modified MJ medium. The ratio of PIPES monosodium salt to PIPES disodium salt was adjusted to keep the medium within the pH range of 6.1−6.3 during culture.

Preparation of the inoculum

Sulfurovum lithotrophicum 42BKT^T (JCM 12117^T) was purchased from the Japan Collection of Microorganisms of the RIKEN Bioresource Center. Cells were precultured in a 125 mL serum bottle (Wheaton Industries, Millville, NJ, USA) containing 20 mL of MJ basal medium and filled with a CO_2/N_2 gas mixture ($p^0_{CO_2}$ = 0.6 atm and $p^0_{N_2}$ = 2.4 atm). The bottle was incubated by shaking at 120 rpm and 29°C (Green Shaker, Vision Scientific Co., Daejeon, Korea). When the optical density of the preculture reached 0.1 at 600 nm, cells were harvested by centrifugation for 7 min at 38,000 *g* and 15°C (SUPRA 30K, Hanil Science Industry, Gangneung, Korea) and washed three times with 30 mL MJ-N synthetic seawater before being used as inoculum.

Biofixation of CO_2

A 125 mL serum bottle was used for culturing under conditions of 1.5 and 3.0 atm, whereas a 120 mL glass-coated stainless steel (SUS 310) reactor equipped with an agitator was used for culturing under a condition of 10 atm (Supplementary Figure S1). Each reactor was inoculated with 0.1 mL bacteria and 60 mL of modified MJ medium. When the serum bottle was closed with a stopper, its inner volume was reduced to 120 mL; hence, the headspace of both reactors was 60 mL.

When CO_2 gas was used as the carbon source, it was supplied as a mixture with N_2 gas in a 2:8 ratio (i.e., $p^0_{CO_2}$: $p^0_{N_2}$ = 2:8). After purging the air in the headspace with the CO_2/N_2 gas mixture for 1 min, the total pressure in the reactor was initially adjusted to 1.5, 3.0, 7.0 or 10.0 atm. $NaHCO_3$ is commonly used as a carbon source in the culture of chemolithotrophs.[31] Hence, $NaHCO_3$ was used as the carbon source in a second series of experiments. $NaHCO_3$ was added at 0.41, 1.31, 4.62 and 8.59 g L^{-1} for cultures under N_2 pressures of 1.5, 3.0, 7.0 and 10.0 atm, respectively. Air replacement and pressure control in the reactor were performed with N_2 only. These concentrations were selected to replicate the concentrations of the CO_2 species in the first series of experiments: 0.41, 1.31, 4.62 and 8.59 g L^{-1} are the concentrations of $NaHCO_3$ corresponding to the total concentrations of CO_2 species (i.e., $[H_2CO_3] + [CO_2(aq)] + [HCO_3^-] + [CO_3^{2-}]$) expected when 60 mL of seawater is in equilibrium with 60 mL (i.e., the headspace volume of the reactors) of CO_2 gas at 0.3, 0.6, and 2.0 atm, respectively. Detailed procedures for the determination of these concentrations are described in Supplementary Information.

The serum bottles were incubated with shaking at 120 rpm; similarly, the rotation speed of the agitator in the stainless steel reactor was maintained at 120 rpm. The culture temperature of both reactors was maintained at 29°C. Culturing was performed for no longer than 24 h, to ensure that the carbon and energy sources in the medium did not become depleted. At the end of the culture periods, the reactors were opened to allow various analyses, such as measurements of cell concentrations, ion concentrations, metabolites, and cellular carbons (Figure S2).

Determination of cell concentrations

Preculture cell concentrations were roughly determined by measuring the optical densities of the culture media at 600 nm using an ultraviolet-visible spectrophotometer (SPECORD 210 PLUS, Analytika Jena, Jena, Germany). More accurate cell concentrations in the CO_2 fixation experiments were also measured, both in terms of number density and dry cell mass concentration, as follows: first, a known volume of a cell culture was filtered through a black polycarbonate Nuclepore filter (0.2 μm pore size, Whatman, Buckinghamshire, UK) placed on top of a cellulose filter (1.0 μm pore size, Advantec MFS Inc., Dublin, CA, USA). To determine the cell number density, the cells on the polycarbonate filter were counted using the acridine orange direct counting method. A microscope equipped with a halogen lamp and a B-2A fluorescence filter block (excitation wavelength: 450−490 nm, barrier wavelength: 520 nm) (Eclipse 6100, Nikon Instruments, Tokyo, Japan) was used in this method.[32, 33] To determine the cell mass concentration, the polycarbonate filter was washed with 30 g L^{-1} NaCl solution and dried in an oven at 30°C until its weight remained constant. A microbalance (CAHN C-35, Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to measure the weight of the filter both before and after it was loaded with cells and dried.

In addition to the above images, scanning electron micrographs of cells were also captured to confirm the influence of CO_2 pressure and amount of $NaHCO_3$ on cell size and morphology (JSM-5410LV, JEOL, Tokyo, Japan).[34]

The cell number densities measured at different culture times yielded growth curves of *Sulfurovum lithotrophicum* 42BKT^T.

Metabolome analysis

Metabolome identification and analysis were performed using a gas chromatograph–mass spectrometer (Agilent 6890 Agilent Technologies, Santa Clara, CA, USA) equipped with a Zebron ZB-1701 column (Phenomenex Inc., Torrance, CA, USA) and a quadrupole mass selective detector (Agilent 5973 MSD, Agilent Technologies, Santa Clara, CA, USA) operated in electron impact mode.

At the end of culturing, cells were harvested by centrifugation for 7 min at 38,000 g and 15°C. The culture supernatant was lyophilized prior to exometabolome analysis. The cell pellet was suspended in 0.5 mL of -70°C aqueous methanol solution (50% v/v) to quench cellular metabolism and initiate the following extraction process: a freeze-thaw cycle of -80°C for 30 min and thawing in an ice bath for 4 min was repeated three times to permeabilize cell membranes and allow leakage of the endometabolome. The suspension was centrifuged for 7 min at 38,000 g and -9°C. The supernatant extract was separated and stored at -80°C, and the pellet was extracted again with 0.5 mL of the methanol solution. The two supernatant extracts were combined and lyophilized at -170°C prior to endometabolome analysis

Prior to their injection into the GC-MS system, lyophilized culture supernatants and extracts were subjected to methyl chloroformate (MCF) derivatization.[35] Each lyophilized sample was added to an 11 mL silanized test tube containing 0.4 mL of 1 M NaOH solution, and mixed with 0.034 mL of pyridine and 0.167 mL of methanol. Then, 0.04 mL of MCF was added twice, and the mixture was vigorously mixed for 30 s after each

addition. Next, 0.4 mL of chloroform was added, and the mixture was vigorously mixed for 10 s to selectively dissolve the MCF derivatives. Then, 0.8 mL of 50 mM $NaHCO_3$ solution was added to the test tubes for endometabolome and exometabolome analyses, respectively. After vigorous mixing for 10 s, the (upper) aqueous layer was allowed to separate from the chloroform layer. The majority of the aqueous layer was discarded using a Pasteur pipette, and 100 mg of anhydrous sodium sulfate was added to completely remove the remaining aqueous layer.

One microliter of the chloroform layer was injected into the GC-MS system, which was operated in pulsed splitless mode.[35] The GC oven temperature was programmed as follows: an initial hold at 2 min at 45°C, a heating step at 9°C min^{-1} to 180°C and then a hold for 5 min, a heating step at 40°C min^{-1} to 240°C and then a hold for 11.5 min, and a heating step at 40°C min^{-1} to 280°C and then a hold for 2 min. Helium gas was used as the carrier gas, at a flow rate of 1.0 mL min^{-1}. The temperatures of the inlet, interface, and quadrupole were 290°C, 250°C, and 200°C, respectively. The GC column was equilibrated for 6 min before each analysis. The mass selective detector was operated in scan mode with a mass range of 38-650 AMU at 1.47 scans s^{-1}.

The Automated Mass Spectral Deconvolution and Identification System (AMDIS, National Institute of Standards and Technology) database was used to identify compounds in the GC spectra by referencing their retention times and mass spectra to those in the MS library of Villas-Bôas et al[35] and our in-house MS library, which was constructed for the components of the culture medium and the expected metabolites of *Sulfurovum lithotrophicum* 42BKT^T. The minimum match factor, the minimum percentage of similarity matching, was set to 75 following the AMDIS manual.

Metabolites were quantified in terms of the response ratio, which was defined in this study as the peak area of a metabolite divided by the peak area of an internal standard.[36] The internal standard was 200 μM of phthalic acid or lysine for the analysis of intracellular or extracellular metabolites, respectively. For each compound identified by GC-MS, standard solutions were prepared with concentrations ranging from 10 to 2000 μM, with 200 μM internal standards.

Analysis of thiosulfate, sulfate, and nitrate ions

Quantitative analysis of thio sulfate, sulfate, and nitrate ions was performed using an ion chromatograph (LC20 Chromatography Enclosure, Thermo Fisher Scientific Inc.) equipped with a conductivity detector. Separation was conducted on an IonPac AS14 column, using a $NaHCO_3/Na_2CO_3$ solution as an eluent at a flow rate of 1.2 mL min^{-1}.

Analysis of cellular carbons

Cells were harvested by centrifugation for 7 min at 38,000 g and 15°C, and then washed 3 times with 30 g L^{-1} NaCl solution. The total amount of cellular carbon was analyzed using an automatic elemental analyzer (EA 1108 CHNS-O, FISONS Instruments, Ipswich, UK).

Specific rates of CO_2 fixation and metabolite formation

The specific rate of CO_2 fixation, q_C, is defined as follows:[37]

$$q_C = -\frac{1}{X}\frac{dS}{dt} = -\frac{1}{X}\frac{dX}{dt}\frac{dS}{dX} = \frac{\mu}{Y_{X/S}} \quad (1)$$

where t is time (hr), S is the concentration of the carbon source (i.e., CO_2 or $NaHCO_3$), X is the dry cell mass, μ is the specific growth rate (hr^{-1}), and $Y_{X/S}$ is the yield coefficient of the carbon source. μ and $Y_{X/S}$ are approximated as:

$$\mu \cong \frac{\ln X_2 - \ln X_1}{t_2 - t_1} \quad (2)$$

$$Y_{X/S} \cong -\frac{\Delta X}{\Delta S} = -\frac{X_2 - X_1}{S_2 - S_1} \quad (3)$$

where X_1 and X_2 denote the dry cell mass concentrations at times t_1 and t_2, respectively. Similarly, S_1 and S_2 denote the concentrations of the carbon source at times t_1 and t_2, respectively. The doubling time, t_d, which is the time required for the cell mass to double, is defined as follows:

$$t_d = \frac{\ln 2}{\mu} \quad (4)$$

During culturing, the inorganic carbon source was consumed and converted to dry cell mass and a very little amount of extracellular metabolites; the change in the concentration of the carbon source (i.e., $-\Delta S$ in Eq. [3]) was thus determined by adding the carbon contents of the dominant extracellular metabolites and the dry cell mass as expressed in terms of molarity (i.e., mole L^{-1}), and multiplying this sum by the molecular weight of CO_2 (44 g mol^{-1}). When $NaHCO_3$ was used as the carbon source, its conversion was also expressed in g CO_2 rather than g $NaHCO_3$ to facilitate comparisons. Hence, the units of S, X, $Y_{X/S}$, and q_C were g CO_2 L^{-1}, g cell L^{-1}, g cell g CO_2^{-1}, and g CO_2 g $cell^{-1}$ hr^{-1}, respectively.

Assuming that the five dominant metabolites in this study were growth-associated products, the specific rate of metabolite formation (mg metabolite g $cell^{-1}$ hr^{-1}), q_P, can be defined as follows:[37]

$$q_P = \frac{1}{X}\frac{dP}{dt} = \frac{1}{X}\frac{dX}{dt}\frac{dP}{dX} = \mu Y_{P/X} \quad (5)$$

where P denotes the metabolite concentration (g metabolite L^{-1}) and $Y_{P/X}$ is the metabolite formation upon the increase in the cell mass (mg metabolite g $cell^{-1}$). $Y_{P/X}$ is approximated as

$$Y_{P/X} \cong \frac{\Delta P}{\Delta X} = \frac{P_2 - P_1}{X_2 - X_1} \quad (6)$$

where P_1 and P_2 denote the metabolite concentrations at times t_1 and t_2, respectively.

Since each metabolite exists in both the interior and exterior of the cells, q_P is divided into two terms as follows:

$$q_P = q_{P,ext} + q_{P,int} \quad (7)$$

where $q_{P,ext}$ and $q_{P,int}$, respectively, denote the specific rates of metabolite formation in the exterior and interior of the cells. Then

$$q_{P,ext} = \mu Y_{P,ext/X} \quad (8)$$

$$q_{P,int} = \mu Y_{P,int/X} \quad (9)$$

where $Y_{P,ext/X}$ and $Y_{P,int/X}$ are the metabolite formations in the exterior and interior of the cells upon the increase in cell mass.

Conclusions

Sustainable and economical fixation of carbon dioxide (CO_2), which is a greenhouse gas most responsible for global warming, has been a topic of much research and development. In this

study, *Sulfurovum lithotrophicum* 42BKT^T, a deep-sea sulfur-oxidizing bacterium, was found to be successful for the biofixation of CO_2 from low (0.3 atm CO_2 with 1.2 atm N_2) to high CO_2 concentration (2 atm CO_2 with 8 atm N_2) at 29°C. Its specific fixation rate was very fast, showing approximately 0.42 g CO_2 g cell^{-1} hr^{-1}. Since 37% of the total amount of CO_2 assimilated as biomass was present in the form of metabolites, mainly glutamate and pyroglutamate, the production possibility of these compounds could be suggested by massive CO_2 biofixation. The cell lengthening was observed at a high CO_2 concentration, but disappeared when the cells were transferred to medium with a low CO_2 concentration. Hence it is believed to be a reversible response to the stress of the high CO_2 level. This work constitutes an important first step toward developing an efficient biofixation strategy of high-concentration CO_2 by using a deep-sea chemolithoautotrophic bacterium. The optimal pressure condition for biofixation can be decided by the type of flue gas supplied, capital and/or operating costs, and site availability. Therefore, the approach used in this study would confer great advantages in terms of flexibility in the engineering process and design, economic evaluation, and energy balance because a wide pressure range of CO_2 containing gas mixtures can be applied for the biofixation of CO_2 under a minute thermal energy penalty. The approach described here constitutes a novel strategy for the biomitigation of CO_2 that is environmentally and economically sound, and therefore has the potential to make a major impact in the global response to climate change.

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Notes and references

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Electronic Supplementary Information (ESI) available: Schematic diagram of the reactor used for the culture at 10 atm, flowchart of CO_2 biofixation experiments and quantitative analyses, images of *Sulfurovum lithotrophicum* 42BKT^T cells grown under different pressures of CO_2 and concentrations of $NaHCO_3$, images of the cells grown under different N_2 pressures but the same concentration of $NaHCO_3$, values of $Y_{X/S}$ and $Y_{P/X}$, carbon fractions in the dry cell masses, and methods for the determination of CO_2 solubility See DOI: 10.1039/b000000x/

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Table of Contents entry

Biological conversion of high-concentration CO_2 in the emission gases using a deep-sea sulfur oxidizing bacterium