Acetyl-CoA carboxylase activity in *Helicobacter pylori* and the requirement of increased CO₂ for growth

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A biotinylated acetyl-CoA carboxylase from the microaerophilic bacterium *Helicobacter pylori* was partially purified and characterized. The approximate molecular mass of the native enzyme was estimated at 235 kDa by native PAGE. A single band corresponding to approximately 24 kDa was detected by SDS-PAGE, suggesting that the native enzyme is a multi-protein complex. The protein was isolated from the soluble fraction of the cell. Catalytic activity was acetyl-CoA-dependent and inhibited by avidin but unaffected by avidin pre-treated with excess biotin. The end-product of the reaction was identified as malonyl-CoA and the reaction was shown to be reversible by NMR spectroscopy. The activity of the enzyme was 0.29 $\mu\text{mol min}^{-1}$ (mg protein)⁻¹. The V_{max} for bicarbonate was calculated at 0.73 $\mu\text{mol min}^{-1}$ (mg protein)⁻¹, and the affinity of the enzyme for this substrate was relatively low, with an apparent K_m of 16.6 mM. These data provide the first evidence of a possible physiological role for the requirement of high levels of CO₂ for growth *in vitro* of this bacterium.

Keywords: *Helicobacter pylori*, acetyl-CoA carboxylase, CO₂

INTRODUCTION

Helicobacter pylori is now recognized as the causative agent of chronic gastritis and a significant proportion of duodenal and gastric ulcers (Marshall & Warren, 1984; Goodwin *et al.*, 1986). Recently, the World Health Organization declared *H. pylori* to be a group 1 carcinogen because of its role in the development of gastric carcinoma (IARC, 1994). Although recent studies have begun to characterize the nutritional requirements of this pathogen (Nedenskov, 1994; Reynolds & Penn, 1994), an understanding of why elevated levels of CO₂ are required by the bacterium for growth has been lacking.

Early studies of the need for CO₂/HCO₃⁻ (in solution at physiological pH the equilibrium favours the formation of bicarbonate) in bacteria that grow at higher partial pressures of CO₂ showed that the compound enters into the synthesis of essential metabolites via carboxylation (Lyman *et al.*, 1947; Ajl & Werkman, 1948). An indication of the probable importance of carboxyl utilization in *H. pylori* physiology was the finding that ¹⁴C-labelled HCO₃⁻ was incorporated during the growth of the bacterium (Mendz *et al.*, 1994b), suggesting the presence of carbamoyl-phosphate synthetase. However, it is difficult to envisage the activity of this enzyme as the sole cause for

the need of high CO₂ concentrations for the growth of *H. pylori*. It is more likely that bicarbonate is required for a number of cellular activities in this micro-organism.

It has been proposed by Cox & Baugh (1977) that some micro-organisms that require an environment enriched in CO₂ may possess enzymes with a high K_m in relation to the specific substrate CO₂/HCO₃⁻; that is, a low affinity for CO₂/HCO₃⁻. Studies of CO₂/HCO₃⁻-metabolizing enzymes in *H. pylori* may reveal such a mechanism or other unique properties of such enzymes that would help us understand the need for an enriched CO₂ environment for growth of this organism *in vitro*. The identification of a biotinylated protein in this bacterium – a group of proteins known to be involved in bicarbonate metabolism in other organisms – presented an opportunity to study one component of CO₂/HCO₃⁻ metabolism in *H. pylori*.

The results presented here report the partial purification and biochemical characterization of acetyl-CoA carboxylase in *H. pylori*. After putatively identifying the protein by size comparisons with other biotinylated proteins, the activity and kinetics of the enzyme were determined in relation to bicarbonate requirements. A preliminary communication of part of this work has been presented previously by Burns *et al.* (1994).

METHODS

Substrates and reagents. $\text{NaH}^{14}\text{CO}_3$ ($0.1 \text{ mCi mmol}^{-1}$; $3.7 \text{ MBq mmol}^{-1}$) was purchased from Amersham Life Sciences. Avidin alkaline phosphatase conjugate, 5-bromo-4-chloro-3-indolyl phosphate and *p*-nitroblue tetrazolium chloride were obtained from Bio-Rad Laboratories. Sephacryl S-300 HR was from Pharmacia. ATP, d-biotin, malonyl-CoA and molecular mass markers were obtained from Sigma, and acetyl-CoA was from Boehringer Mannheim. Blood Agar Base no. 2 was from Oxoid. All other reagents were of analytical grade.

Strains and growth conditions. *H. pylori* strains NCTC 11639, UNSW P10, UNSW RU1, N6 and a recent clinical isolate, strain 921007, were grown on Blood Agar Base no. 2 plates, supplemented with 5–7% (v/v) horse blood. The medium was supplemented with fungizone ($2 \mu\text{g ml}^{-1}$), trimethoprim ($5 \mu\text{g ml}^{-1}$), polymyxin B ($2.5 \mu\text{g ml}^{-1}$) and vancomycin ($10 \mu\text{g ml}^{-1}$). *H. pylori* cultures were incubated in a Stericult incubator (Forma Scientific) in an atmosphere of 10% CO_2 in air, 95% relative humidity, at 37°C . Cells were passaged every 36–48 h, checked for purity under phase-contrast microscopy and tested for positive urease and catalase activity.

Preparation of cell-free extracts. Cells were harvested in exponential phase (approximately 36 h) in 0.1 M sodium phosphate buffer, pH 7.2 (PB), and centrifuged at $17000 g$ for 8 min at 6°C . The resulting pellet was washed twice and resuspended in approximately 5 ml PB. Cells were disrupted by two cycles through a French pressure cell, or by twice freezing in liquid nitrogen and thawing cell suspensions. The lysed extract was then centrifuged at $27000 g$ for 8 min at 6°C . The resulting supernatant was filtered through a $0.2 \mu\text{m}$ pore-size membrane filter and the filtrate collected. Unless otherwise stated, this filtrate represents the 'cell-free extract' described in later sections.

Cell fractionation. To investigate the cellular location of the biotinylated protein, *H. pylori* cell-free extracts were centrifuged at $100000 g$ for 60 min at 4°C to separate the membranes from the cytosol. The supernatant was removed, and the resulting membrane pellet was resuspended in PB. All bacterial fractions were stored at -20°C until required.

Protein determination. Protein concentrations were estimated by the bicinchoninic acid method using a microtitre-plate-based protocol (Pierce Chemical).

Purification of biotinylated protein. The cell-free extract (approximately 3.5 mg ml^{-1}) was applied to a K 26/100 gel-filtration column containing Sephacryl S-300 HR. A liquid phase of PB at a flow rate of 1.2 ml min^{-1} was used to elute proteins. Fractions were taken at 4 min intervals and an elution profile was obtained by scanning the eluate at 280 nm with a Vicord 2138 spectrophotometer (LKB Bromma).

Column fractions testing positive for biotinylated proteins using the avidin-linked Western blot were pooled, then concentrated by ultrafiltration through a 100 kDa molecular mass cut-off filter (Millipore) using an Amicon 8050 filtration unit. Proteins retained by the filter were resuspended by washing off the filter in PB, and the filtrate passed through a 30 kDa cut-off membrane filter (Amicon). The proteins retained on this filter were resuspended in PB, and the filtrate and retentate from each step were analysed for the presence of biotinylated proteins.

A monomeric avidin column was prepared and the protein samples were loaded and eluted according to Beaty & Lane (1982) and Gravel *et al.* (1980) using an Affinity Pak of immobilized avidin (Pierce).

SDS-PAGE. Electrophoretic analysis of fractionated proteins was performed using a discontinuous buffer system, based on an established procedure (Laemmli, 1970). Protein samples (approximately 1 mg ml^{-1}) were denatured and reduced by mixing in a sample buffer that contained 2% (v/v) SDS and 0.05% (v/v) β -mercaptoethanol then heated in a boiling water bath for 10 min. Gels were initially stained with Coomassie Blue; however a more sensitive detection of proteins was achieved by employing a rapid silver stain (Neilan *et al.*, 1994). Proteins of known molecular size, bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa) and trypsin inhibitor (20.1 kDa), were used to construct a standard curve.

Native molecular mass determination by electrophoresis.

Non-reducing and non-denaturing gel electrophoresis was performed by modifying the procedure of Laemmli (1970). Separating gels were composed of 8% (w/v) acrylamide and SDS was omitted from all solutions. Protein mixtures were not boiled or reduced and samples were electrophoresed as for SDS-PAGE. Markers were: β -amylase (200 kDa), alcohol dehydrogenase (150 kDa), β -galactosidase (116 kDa), phosphorylase B (97.4 kDa), bovine albumin (66 kDa) and egg albumin (45 kDa).

Western blotting. After electrophoresis, proteins were transferred to a blotting membrane in a semi-dry electroblotting (SDE) transfer system. Transfer was carried out in a Milliblot-SDE Transfer System (Millipore), based on an established protocol (Towbin *et al.*, 1979). After transfer, membrane-bound biotinylated proteins were detected utilizing an avidin-linked enzyme conjugate. Unoccupied protein-binding sites on the membrane were first blocked in TBS (20 mM Tris, 500 mM NaCl, pH 7.5) containing 3% (w/v) gelatin. The membrane was then washed in 0.05% (v/v) Tween-20 TBS (TTBS) for 10 min. The blot was incubated for 1 h with avidin alkaline phosphatase conjugate (1:1000) in TTBS containing 1% gelatin. The membrane was then washed three times (10 min each) in TTBS, followed by two washes (5 min each) in distilled water. After the final wash, 0.56 mM 5-bromo-4-chloro-3-indolyl phosphate and 0.48 mM *p*-nitroblue tetrazolium chloride were added to 10 mM Tris/HCl (pH 9.5) and incubated with the membrane until sharp purple bands appeared. Substrate development was stopped by rinsing in distilled water.

Radioactive tracer analysis. The acetyl-CoA carboxylase assay method used was based on that of Gregolin *et al.* (1966), and measured the incorporation of [^{14}C]bicarbonate into an acid-stable product, malonyl-CoA. The assay mixture contained the following components: ATP, 1 mM; $\text{NaH}^{14}\text{CO}_3$, 10 mM; MgCl_2 , 1 mM; EDTA, 1 mM; acetyl-CoA, 1 mM; BSA, 2 mg ml^{-1} (measurements at this concentration of bicarbonate were used to demonstrate enzyme activity before kinetic studies were conducted). The assay mixture was made to a final volume of 200 μl with PB (pH 7.0). The reaction was initiated by adding either *H. pylori* lysates or cell-free extracts. Mixtures were incubated for 60 min at 37°C .

The kinetics of the enzyme were studied by varying the concentration of bicarbonate and keeping acetyl-CoA and ATP saturating and constant (1 mM). The data were fitted to the Michaelis-Menten equation using the program Enzyme Kinetics (Trinity Software), where the values of the kinetic parameters and standard errors are automatically calculated. Reactions were terminated by the addition of formic acid (0.1 ml, 3 M) and solutions dried at 80°C for 15 min, during which time any unreacted $\text{H}^{14}\text{CO}_3^-$ was evaporated as $^{14}\text{CO}_2$. Ten millilitres of scintillation fluid (0.5%, w/v, 2,5-diphenyloxazole toluene) was

added, and the radioactive decay measured on a Packard Tricarb scintillation counter.

NMR spectroscopy. Acetyl-CoA carboxylase activity was investigated employing ^1H -NMR, where the fate of specific metabolites could be followed over time. For NMR measurements, lysates, supernatants or membranes were resuspended in 150 mM NaCl constituted in $^2\text{H}_2\text{O}$ and dispensed in 5 mm tubes (Wilmad). Free induction decays were collected using a Bruker AM-500 spectrometer, operating in the Fourier transform mode. Measurements were carried out at 37 °C. Sequential spectra were acquired automatically at 500.11 MHz with pre-saturation of the water resonance.

The instrumental parameters were: spectral width 5434.7 Hz, memory size 8 K, recycling time 3.5 s, number of transients 256, and pulse angle 50° (8 μs). Exponential filtering of 1 Hz was applied prior to Fourier transformation. Substrate concentrations for the reaction were: 20 mM acetyl-CoA, 40 mM ATP, 40 mM NaHCO_3 and 5 mM MgCl_2 . Concentrations are different to those used in the radioactive tracer assay as higher levels of assay components are needed to detect and distinguish reactants in an NMR spectrum. The reaction was started by adding 100 μl bacterial preparation to a total sample volume of 500 μl .

The time-evolution of products and utilization of substrates was followed by acquiring sequential spectra. Assignment of resonances was achieved by adding the appropriate metabolite and comparing its spectral position with that of the products in the bacterial preparation.

RESULTS

Identification and partial purification of a biotinylated protein in *H. pylori*

After extensive investigation of bacterial extracts, it was found that there was only a single biotinylated product present in *H. pylori*. A high molecular mass biotinylated protein was identified in cell-free extracts of all strains tested. When cell-free extracts were applied to a Sephacryl S-300 HR column, the biotinylated protein was detected in fractions eluted just after the void volume (Fig. 1). Fractions testing positive for this protein by Western

Fig. 1. Elution profile of *H. pylori* cell-free extracts obtained by gel filtration through a Sephacryl column. Proteins were eluted with 100 mM sodium phosphate buffer at a flow rate of 1.2 ml min^{-1} and detected at 280 nm (○). ●, Fractions testing positive for biotinylated proteins.

Fig. 2. Size characterization of *H. pylori* biotinylated protein. Samples obtained after membrane filtration were denatured and reduced, then electrophoretically separated using SDS-PAGE. The Western blot of this gel was then probed with an avidin-linked conjugate and analysed for the presence of biotin. Lanes: 1 and 2, all soluble proteins; 3 and 4, proteins greater than 100 kDa in size; 5 and 6, proteins less than 100 kDa; 7 and 8, proteins 30–100 kDa; 9 and 10, proteins less than 30 kDa.

blotting were subsequently pooled. The biotin-containing protein from this semi-pure extract was further characterized by membrane filtration. SDS-PAGE of the retentate and filtrate and subsequent Western blotting showed that the biotinylated protein was retained only in fractions containing molecules greater than 100 kDa in size (Fig. 2).

Purification of the biotinylated protein using the monomeric avidin column was not successful. It was found that the protein was only partially retarded on the column, and the recovery of protein was poor. Other authors have encountered similar problems in the purification of biotinylated proteins, particularly in relation to the elution of these proteins from avidin columns (Berger & Wood, 1975; Swack *et al.*, 1978).

Determination of cellular location of acetyl-CoA carboxylase

When crude cell-free extracts were separated into supernatant and membrane fractions the biotinylated protein was isolated in the soluble fraction. Although the protein was found to be associated with this fraction, it is not known whether the enzyme is cytoplasmic or periplasmic.

Molecular mass determinations

To determine the molecular mass of the native protein complex, samples were analysed with PAGE under non-denaturing and non-reducing conditions. Attempts to estimate the protein's size by analytical gel filtration were not productive. Gel filtration yielded broad peaks unsuitable for accurate protein size determination (range ~190–280 kDa). For this reason we opted instead for native PAGE. The presence of a single biotinylated band by this method enabled us to estimate molecular size.

Table 1. Demonstration of acetyl-CoA and biotin dependencies on acetyl-CoA carboxylase in *H. pylori* cell-free extracts

The concentrations of assay components were: 1 mM acetyl-CoA; 10 mM HCO_3^- ; 1 mM ATP; 1 mM Mg^{2+} ; 0.2 mg BSA. Assay mixtures were incubated at 37 °C for 60 min. Values are expressed as means of at least duplicates; 100% activity is equivalent to the carboxylation of $0.307 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$.

Conditions	Carboxylase activity (%)
Complete reaction mixture	100
Without enzyme extract	2.6
Without acetyl-CoA	12.4
With avidin	6.3
With biotin-saturated avidin	87.9

From the relative mobility of the enzyme and from those of the standard proteins, a calibration curve was constructed where the native molecular size was estimated at 235 ± 16 kDa. Employing denaturing and reducing SDS-PAGE, a biotinylated protein subunit of molecular mass 24 ± 2.1 kDa was obtained (Fig. 2).

Identification of enzyme activity

Comparisons of the size of the protein complex and subunit in *H. pylori* with known bacterial biotinylated proteins suggested the possibility that the enzyme was acetyl-CoA carboxylase. An acetyl-CoA-dependent carboxylase activity was identified in *H. pylori* extracts (Table 1). Cell-free extracts showed acetyl-CoA carboxylase activity at a rate of $0.285 \pm 0.024 \mu\text{mol product formed min}^{-1} (\text{mg protein})^{-1}$, with a linear activity for 75 min under the experimental conditions employed. The enzyme preparation was incubated for 10 min with 2 mg avidin ml^{-1} to determine whether the observed activity was from a biotinylated protein. Catalysis of $\text{H}^{14}\text{CO}_3^-$ was almost completely inhibited when avidin was present in the assay system; however activity was unaffected when avidin was pre-treated with 10 mM biotin (Table 1). These results provided reliable evidence for a biotin-dependent carboxylase activity in *H. pylori*.

End-product identification

$^1\text{H-NMR}$ spectra showing the time-evolution of substrates and products of the acetyl-CoA carboxylase reaction in incubations with *H. pylori* cell-free extracts are shown in Fig. 3. The decrease in the peak of the methyl group of acetyl-CoA and the appearance of a peak corresponding to malonyl-CoA was observed. The assignment of this peak was carried out by comparing its chemical shift with that of malonyl-CoA. Hydrolysis of acetyl-CoA was also present in these preparations (Fig. 3), yielding acetate and CoA as products. The production of

Fig. 3. $^1\text{H-NMR}$ spectra of a time-course of acetyl-CoA carboxylation by *H. pylori* cell-free extracts. The resonances corresponding to the substrate acetyl-CoA and the carboxylation product malonyl-CoA are indicated on the figure. The inset corresponds to enlargements of the malonyl-CoA peak. Resonances corresponding to free CoA and acetate are also shown. The times at which each spectrum was acquired are shown on the right-hand side.

malonyl-CoA and the hydrolysis of acetyl-CoA was shown to be enzymic and not chemical, as none of these changes were observed when the substrates were incubated without enzyme preparation.

Kinetic studies

For kinetic analysis, carboxylation of acetyl-CoA was studied over a concentration range of 0.5–50 mM for bicarbonate employing radioactive trace analysis, with both acetyl-CoA and ATP at a concentration of 1 mM. The latter substrates were shown to be saturating as no increase in activity was observed when the concentrations were raised to 5 mM and 10 mM for acetyl-CoA and ATP, respectively. The enzyme reaction as a function of bicarbonate concentration was saturable, with a V_{max} of $0.730 \pm 0.048 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$. The apparent K_m value of the enzyme for bicarbonate was at 16.6 ± 2.7 mM.

DISCUSSION

A biotin-containing acetyl-CoA carboxylase system from *H. pylori* has been identified and partially characterized, with the focus on the efficiency of carboxylation. The present investigation utilized the high affinity of avidin for biotin ($K_d = 10^{-15}$ M; Green, 1963) to detect biotinylated enzymes in a heterogeneous population of proteins. In this study, the characteristics of the biotinylated protein in *H. pylori* could be studied in cell-free extracts, as the presence of the enzyme could be detected specifically on a Western blot using an avidin-linked enzyme conjugate. The demonstration of acetyl-CoA-dependent enzyme activity and the product formation of malonyl-CoA identified this protein as an acetyl-CoA carboxylase, and a central role for biotin was indicated by avidin-inhibited carboxylase activity.

The acetyl-CoA carboxylases of bacteria are commonly multi-enzyme complexes and not just multi-functional proteins as in the eukaryotic equivalents (Moss & Lane, 1971). The bacterial enzymes studied to date have been ternary complexes with each component having a distinct function in acetyl-CoA carboxylation. One component is a biotinyl CO_2 carrier protein involved in carboxyl transfer between substrates (Knowles, 1989). The demonstration in the present study of a biotinylated protein component of 24 kDa corresponded very well with the molecular mass (22.5 kDa) of the biotinyl CO_2 carrier protein of *Escherichia coli* acetyl-CoA carboxylase described by Guchhait *et al.* (1974). It also correlated with the 25 kDa protein associated with acetyl-CoA carboxylase in *Pseudomonas citronellolis* (Ray & Fall, 1976). Other similarities were also seen between the native size of the acetyl-CoA carboxylase in *E. coli* and *H. pylori*. An approximate molecular mass of 235 kDa was measured for the complex in *H. pylori*, while in *E. coli* the native complex exhibited a size of 250 kDa (Guchhait *et al.*, 1974). These findings suggest that the 24 kDa biotinylated band visualized in SDS-PAGE preparations of *H. pylori* may be the biotinyl CO_2 carrier protein of the acetyl-CoA carboxylase complex.

One feature of the *H. pylori* biotin protein that appeared to be different to *E. coli* is the strength of the interactions between the proteins in the complex. *E. coli* acetyl-CoA carboxylase is completely dissociated under most purification conditions (Guchhait *et al.*, 1974). It was observed, however, that the complex in *H. pylori* is relatively stable through most stages of purification, as the protein testing positive for biotin was always retained on the 100 kDa molecular mass cut-off filter (Fig. 2). This result is similar to the finding in *Propionibacterium shermanii* (Stirling *et al.*, 1981), which also has a relatively stable acetyl-CoA carboxylase.

NMR spectroscopy in this study was employed to support the radioactive tracer analysis data and to identify the end-product malonyl-CoA. The data showed that acetyl-CoA utilization occurred faster than malonyl-CoA formation. The dramatic reduction in the acetyl-CoA peak can be explained by the cleavage of the high-energy acetyl-CoA bond to form acetate and free CoA, both of which

increased in the sequential spectra (Fig. 3). Furthermore, as these results were obtained using cell-free extracts other enzyme reactions may also be occurring. Acetyl-CoA is also required in the next step of fatty acid synthesis in the formation of an acetyl-acyl carrier protein complex (Stryer, 1988), and the presence of phosphotransacetylase in the fermentation of pyruvate has been established previously in *H. pylori* (Mendz *et al.*, 1994a).

The slow increase in the malonyl-CoA peak could also be attributed to a rapid turn-over of this product as malonyl-CoA is metabolized in the next step of fatty acid synthesis, and is also one of the intermediates in the *de novo* formation of biotin (Lezius *et al.*, 1963). It is not known whether *H. pylori* has the capability to synthesize its own biotin, and it would be interesting to determine whether the product of a biotin-dependent reaction was involved in the formation of biotin itself. Another enzyme involved in the hydrolysis of malonyl-CoA is a decarboxylase, present in *Pseudomonas fluorescens* (Hayaishi, 1955), which may also contribute to malonyl-CoA loss if present in *H. pylori*.

The Michaelis constant of the enzyme for bicarbonate revealed that the enzyme has a very low affinity for bicarbonate. A K_m of 16.6 mM shows that the *H. pylori* enzyme has a lower affinity for HCO_3^- than acetyl-CoA carboxylases reported for other micro-organisms: 2.9 mM for *E. coli* (Guchhait *et al.*, 1974) and 3.0 mM for *P. shermanii* (Stirling *et al.*, 1981). Unlike *H. pylori*, these organisms do not have a requirement for elevated levels of CO_2 , suggesting that the high K_m is related to the ecological niche of this bacterium.

From an evolutionary point of view, the question arises as to whether in colonizing its ecological niche the bacterium evolved with a low affinity enzyme or developed it as an adaptation to this unique environment. It has been shown that there is a significant amount of HCO_3^- in the normal gastric mucus (Gamble, 1950), and the activity of *H. pylori* urease may also contribute to an increased availability of bicarbonate (Hazell, 1992). Certainly urease activity appears to be essential for colonization (Eaton *et al.*, 1991). Thus both the host and the bacterium may contribute to the creation of an environment with an increased bicarbonate concentration. In such an *in vivo* environment acetyl-CoA carboxylase with a low affinity for the substrate may function quite efficiently. Indeed an enzyme with a low affinity for the substrate may be desirable in such circumstances.

In summary, this study has identified the first committed step in *de novo* fatty acid synthesis in *H. pylori*, and as such the enzyme could be expected to be essential for the organism. In addition, the enzyme was detected in all strains tested, including a low passage clinical isolate, and results from the kinetic experiments suggest that acetyl-CoA carboxylase of *H. pylori* can carboxylate acetyl-CoA efficiently only at high concentrations of bicarbonate. It is possible that the kinetic characteristics of this enzyme are one of the causes of the requirement of an increased CO_2 or bicarbonate environment for growth of *H. pylori in vitro*. To obtain a complete understanding of the role of $\text{CO}_2/\text{HCO}_3^-$ in the physiology of the bacterium, it will be

necessary to study other enzymes in this bacterium that utilize these metabolites.

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