AUTOTROPHY IN IRON-OXIDIZING, ACIDOPHILIC BACTERIA.

by

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This thesis is presented for the
Degree of Doctor of Philosophy,
in the Department of Biological Sciences,
University of Warwick.

April, 1993.

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Acknowledgements

I would like to thank my supervisor, Dr. Paul Norris, for his enthusiasm and constructive input throughout the course of this project.

Thanks also to my parents and brother for whose continual support and encouragement I am eternally grateful.

I would like to thank all members of the Microbiology I research group for providing a pleasant working atmosphere, and in particular Jim Trickett, whose contribution will be sadly missed. Thanks also to John, Tim, Darren, Jo, Howard and Heather.

I acknowledge the support of the Science and Engineering Research Council and Norman Le Roux (DTI Warren Springs Laboratory) for this CASE studentship.

Declaration

I declare that this thesis has been composed by myself and has not been used in any previous application for a degree. All results presented within have been obtained by myself under the supervision of Dr. Paul Norris.

Abbreviations

A	absorbance
AMP	adenosine monophosphate
AMPS	ammonium persulphate
ADP	adenosine diphosphate
ATP	adenosine triphosphate
B.G.	back ground
B.S.A.	bicarbonate specific activity
C_n	compound containing n carbon atoms
C.A.	carbonic anhydrase
Ci	Curie
C_i	inorganic carbon
CCM	carbon concentrating mechanism
cpm	counts per minute
CTAB	cetyltrimethylammonium bromide
Da	Dalton
DNA	deoxyribonucleic acid
E.A.	enzyme activity
EDTA	ethylene(diamino)tetra acetate
g	gramme
x g	gravitational force
g/L	grammes per litre
G+C	guanosine + cytidine
kb	kilobase
mol	mole
NAD(P)	nicotinamide adenine dinucleotide (phosphate)
NAD(P)H	reduced form of NAD(P)
O.D.	optical density
PAGE	polyacrylamide gel electrophoresis
PEP	phospho(enol) pyruvate
pers. comm.	personal communication
P_i	orthophosphate
PRK	phosphoribulokinase
RNA	ribonucleic acid
RuBisCO	ribulose bisphosphate carboxylase/oxygenase
RuBP	ribulose-1,5-bisphosphate
SDS	sodium dodecyl sulphate
TCA	tricarboxylic acid
TEMED	tetramethylethylene diamine
v/v	volume per volume
w/v	weight per volume

Abstract

Gram positive, moderately thermophilic, acidophilic bacteria which can oxidize mineral sulphides are likely to be used industrially in mineral processing. In comparison with the Gram negative *Thiobacillus ferrooxidans* which is currently used commercially, the best-characterized moderate thermophiles were shown to require enhanced concentrations of CO_2 in air (i.e. greater than 0.033% (v/v)) for optimal growth. The influence of the supplied CO_2 concentration was examined with growth of moderate thermophile strain BC1 and of *T. ferrooxidans* with ferrous iron as the substrate. Various aspects of CO_2 utilization by these bacteria were examined with a view to explaining the requirement of the Gram positive thermophiles for a higher CO_2 concentration.

During autotrophic growth under CO_2-limitation (i.e. aeration with air, 0.033% (v/v) CO_2), strain BC1 and *T. ferrooxidans* both showed an approximate three-fold increase in RuBisCO synthesis and were capable of similar maximum RuBisCO and PRK activities. RuBisCO substrate affinities (K_m) for strain BC1 were 43.5 µM (CO_2) and 67.5 µM (RuBP) and for *T. ferrooxidans* were 47 µM (CO_2) and 85 µM (RuBP), again apparently indicating no major differences in the activity of these Calvin cycle enzymes. Both types also appeared to possess only the form I RuBisCO (L_8S_8).

T. ferrooxidans was shown to induce a high affinity CO_2 uptake system following growth under CO_2-limitation, which allowed the bacteria to show a maximum rate of CO_2 uptake from air (0.03% (v/v) CO_2). The moderate thermophile strains BC1 and ALV did not induce a high affinity system under CO_2 limitation, and required 0.1% (v/v) CO_2 in air for the maximum rate of CO_2 uptake, which was a similar maximum rate to that of *T. ferrooxidans*.

After harvesting and resuspension, strains BC1 and ALV, but not *T. ferrooxidans* had an obligate requirement for a "regeneration" period in the presence of an energy source (ferrous iron) before CO_2 uptake could be demonstrated. This period may have been necessary for *de novo* protein synthesis.

The presence of some unfixed carbon in the strain BC1 cytoplasm possibly indicated a less efficient utilization of accumulated CO_2 in comparison with *T. ferrooxidans*. The presence of carboxysomes in *T. ferrooxidans* could have contributed to the efficient CO_2 utilization; they were not observed in the Gram positive moderate thermophiles.

Three membrane polypeptides (approximate molecular weight 93, 62 and 35 kDa) and two apparently soluble and potentially acid-stable polypeptides appeared to increase in concentration in *T. ferrooxidans* following growth under CO_2-limiting conditions.

A recently obtained enrichment culture of moderate thermophiles from an Icelandic hot spring was found to be capable of relatively efficient growth on ferrous iron under air, in comparison with strain BC1. Electron microscopy revealed that attempts to isolate a pure culture which was capable of such growth had left a culture which comprised two types of bacteria. One of these bacteria appeared to be morphologically similar to strain BC1 whereas the other appeared to be a previously undescribed organism. A harvested and resuspended mixture of these two types (from growth under CO_2-limitation) possessed a high affinity CO_2 uptake system (kinetically equivalent to that of *T. ferrooxidans*). The relative contribution of these two types to the efficient CO_2 utilization awaits their separation. The correlation of good growth under air with the presence of high affinity transport systems, rather than with different "efficiencies" of CO_2 fixation in the Calvin cycle, nevertheless appears established for the iron-oxidizing acidophiles.

CHAPTER 1

INTRODUCTION

1.1 Summary of project aims

The object of this study was to investigate carbon dioxide (CO_2) utilization by Gram positive, acidophilic, moderately thermophilic, iron- and mineral sulphide-oxidizing bacteria during autotrophic growth. Strain BC1 (see section 1.2 and 1.2.2) was the bacterium used for most of the work. There were two main reasons for this study.

Firstly, the bacteria in question are of potential industrial use but their utility could be diminished by an apparent deficiency with regard to carbon assimilation in comparison with most other autotrophs, i.e. they appear to have a "higher than air" (greater than 0.033% (v/v)) CO_2 concentration requirement for rapid growth. This study was to determine the reason, or reasons, for this "deficiency" which may then allow consideration of the possibility for strain improvement.

Secondly, the autotrophic growth of Gram positive bacteria appears not to have been studied in detail.

Because of its known capacity for efficient autotrophic growth with air levels of CO_2 (0.033% (v/v)), *Thiobacillus ferrooxidans* was used as a "control" organism throughout this study, i.e. as an organism which would have allowed confirmation of the efficacy of the techniques used or developed to illustrate the presence, absence or activities of key aspects of CO_2 utilization in the relatively poorly studied iron-oxidizing thermophiles. The following aspects of CO_2 utilization have been considered:

i) Presence and activity of CO_2-fixing enzymes

ii) Characteristics CO_2-fixation enzymes (i.e. substrate affinities)

iii) Characteristics of CO_2 uptake

iv) The possible involvement of carboxysomes

1.2 The mineral sulphide-oxidizing bacteria and bacterial mineral leaching

The industrial application of bacterial involvement in the solubilization of metals from mineral sulphides (for reviews see Lundgren and Silver, 1980; Hutchins *et al.*, 1986; Brierley and Brierley, 1986; Kelly 1988) has recently been extended beyond the relatively crude dump and heap operations for copper and uranium recovery to the use of bioreactors in the leaching of precious metals from refractory ores (see Hutchins *et al.*, 1987; Spencer *et al.*, 1989). These bioreactors offer a major advantage over the more traditional "open" methods by allowing much greater control over the conditions affecting bacterial growth and activity.

A few such bioreactors are now operational globally and include those at the Genmin Fairview mine, South Africa and the Harbour Lights mine, Western Australia. The daily handling capacity of these reactors are 18 and 40 tonnes respectively.

The reactions involved in mineral leaching are complex and include the direct bacterial attack on the sulphide moiety of the ore resulting in sulphuric acid production and the indirect chemical attack by ferric iron, which itself is recycled through bacterial oxidation of the ferrous iron (for reviews see Lundgren and Silver, 1980; Hutchins *et al.*, 1986).

Prior to 1972 *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* were the sole micro-organisms that had been described as involved in bioleaching (Kelly, 1988). However, since then an abundance of diverse iron- and sulphur-oxidizing bacteria have been identified from a variety of acidic, mineral sulphide environments (for reviews see Norris, 1989a and 1990; Brierley and Brierley 1986).

These bacteria can be divided into three groups according to their optimum growth temperature: the mesophiles, the moderate thermophiles and the extreme thermophiles; with characteristic temperature optima of 30°C, 50°C and 70°C respectively. Of these groups, the thermophiles have recently attracted increasing commercial interest. This interest has resulted from the faster mineral dissolution rates associated with higher temperatures (see Fig. 1.1 and Marsh and Norris 1983b), in addition to a reduced cooling requirement: a consequence of the exothermic reactions involved in bioleaching (Hutchins *et al.*, 1987). Spencer *et al.* (1989) described a possible application of a moderately thermophilic bacterial culture for the treatment of a refractory arsenopyrite concentrate. The higher temperature and fluctuations (30 to 49°C) experienced within their reactor suited the use of this group of bacteria in preference to a mesophilic culture.

It is interesting to note that the temperature basis of the three groupings also appears to reflect some major phylogenetic divisions. Although considerable diversity probably exists within the mesophiles with respect to iron oxidation systems (see Norris, 1989a) and mineral-oxidizing capacities (see section 1.2.1), all isolates to date appear to be Gram negative. In contrast, the iron- and sulphur-oxidizing moderate thermophiles are Gram positive and the extreme thermophiles all belong to the kingdom of archaeabacteria.

Because of this phylogenetic diversity, certain physiological and biochemical features such as mechanisms of iron oxidation and possibly CO_2 fixation pathways appear to be group specific (Norris, 1990). Consequently, the factors that may limit the potential application of these different bacterial groups are diverse and include the CO_2 concentration, metal toxicity, ferric iron inhibition and sensitivity to agitation with high pulp densities.

The CO_2 concentration is of some commercial significance in considering application of the moderate thermophiles. Fig. 1.1 illustrates the requirement that this group of bacteria (typified by strain BC1) have for elevated levels of CO_2 in air in order to support a high rate of mineral dissolution.

Under conditions of 1% (v/v) CO_2, the extreme thermophile *Sulfolobus* and moderate thermophile strain BC1 exhibit a two- to three-fold faster solubilization rate than the mesophile *T. ferrooxidans*. However, when this CO_2 concentration was decreased to the level found in air, the associated iron solubilization rate for the moderate thermophile was reduced to below that for *T. ferrooxidans*. In contrast, at air levels and above, the CO_2 concentration had little effect on the activities of either the mesophile or the extreme thermophile.

1.2.1 The mesophilic iron- and sulphur-oxidizing bacteria

Thiobacillus ferrooxidans is the most extensively studied bacterium in mineral leaching and is often regarded as a standard with which other potentially useful micro-organisms can be compared. This iron- and sulphur-oxidizing, rod-shaped bacterium (approximately 0.5 by 1.5 μm) sometimes possesses a polar flagellum and has an optimum growth temperature of between 30 and 35°C. *T. ferrooxidans* has been generally considered to be an obligate autotroph, although glucose utilization has been noted (Barros *et al.*, 1984), and like all thiobacilli (Karagouni and Kelly, 1989), primarily obtains its cellular carbon from CO_2 via the Calvin cycle. Phosphoenolpyruvate carboxylase has been purified from this organism (Din *et al.*, 1967), but like that from *T. thiooxidans* (Howden *et al.*, 1972), does not contribute notably to primary cellular carbon fixation.

Another mesophile, *Leptospirillum ferrooxidans*, exhibits a greater tolerance towards increasing ferric:ferrous iron ratios (Norris *et al.*, 1988) and acidity (see Norris, 1990). These advantages may account

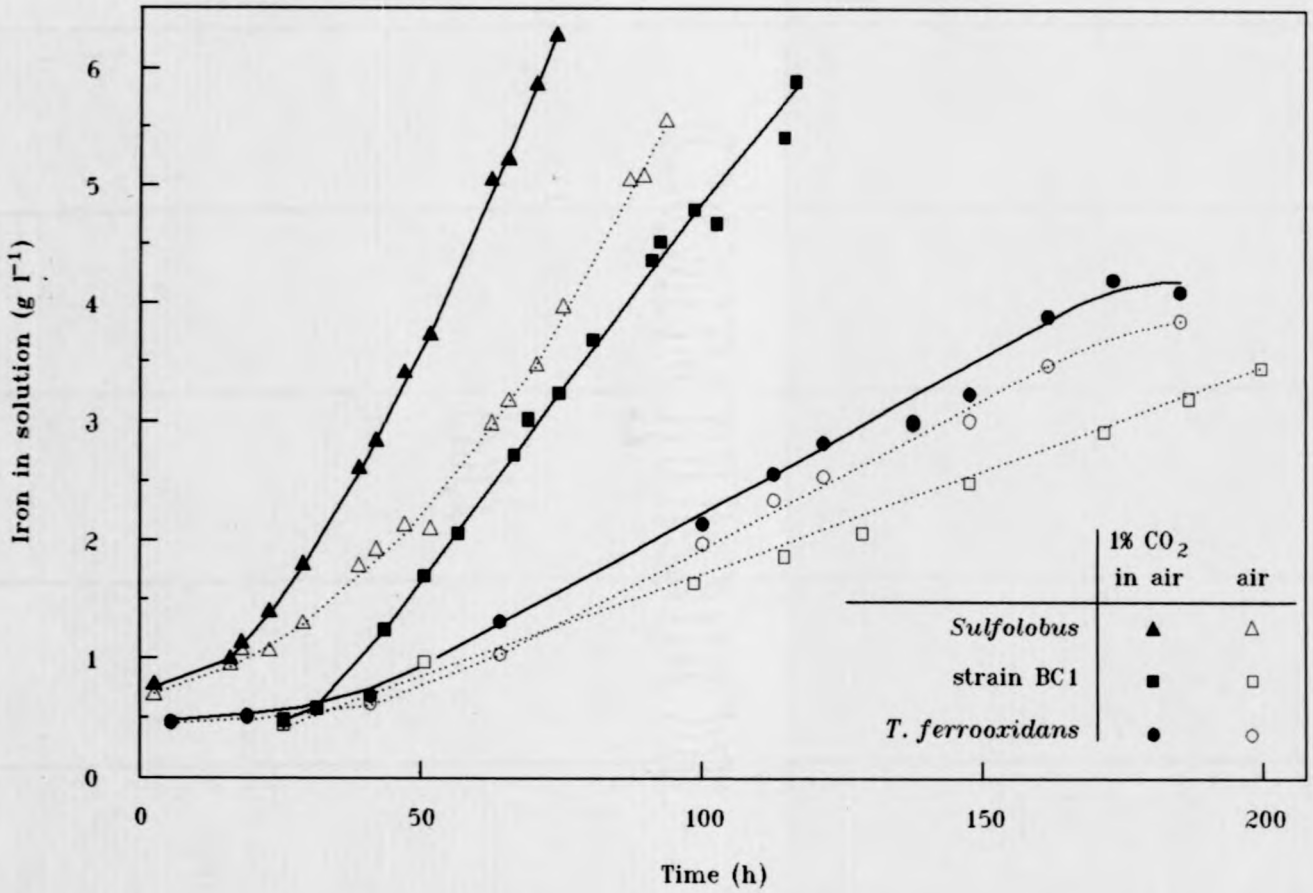


Fig. 1.1 Dissolution of pyrite (FeS_2) by bacteria in air-lift reactors gassed with air or CO_2-enriched air at 30°C (*T. ferrooxidans*), 50°C (strain BC1) and 70°C (*Sulfolobus*). From Norris, 1989b.

for the ability of this organism to compete successfully with *T. ferrooxidans* during growth on pyrite. However, the inability of *L. ferrooxidans* to oxidize elemental sulphur probably restricts its application in the leaching of high sulphur containing ores (e.g. chalcopyrite) without complementation by a sulphur-oxidizing bacterium (see Norris, 1990).

The lack of an iron-oxidizing capacity of *T. thiooxidans* probably restricts the activity of this organism in pure culture to certain minerals, particularly zinc and cadmium sulphides (see Norris, 1990). However, successful dissolution of chalcopyrite has been achieved by *T. thiooxidans* as a mixed culture with *L. ferrooxidans*. The former organism was responsible for removal of a sulphur passivation layer which would otherwise have prevented dissolution by a pure culture of *L. ferrooxidans* (Norris, 1990).

Two more recent isolates *Thiobacillus phimbophilus* (Drober *et al.*, 1992) and *Thiobacillus cuprinus* (Huber and Stetter, 1990) have been initially characterized though not yet used in metal processing studies. The latter organism, like *T. thiooxidans*, is devoid of an iron-oxidizing capacity (Huber and Stetter, 1990).

Other mesophilic, mineral sulphide-oxidizing, acidophiles such as *Thiobacillus albertis*, *Thiobacillus acidophilus* and various species of *Acidiphillium* have been identified (see Norris, 1990), but only poorly studied.

1.2.2 The moderate thermophiles

A variety of Gram positive, acidophilic iron- and sulphur-oxidizing bacteria with temperature optima around 50°C have been isolated. These include *Sulfolobus thermosulfidooxidans* isolated in eastern Europe (Karavaiko *et al.*, 1988); strain BC1 and strain ALV isolated in Warwickshire, U.K. (Marsh and Norris, 1983a); strain TH3 isolated from

a copper mine in New Mexico (Brierley, 1978); and strain NMW-6 isolated in New South Wales, Australia (see Holden, 1991).

It is apparent from the 16S rRNA work of Lane *et al.* (1992) that these bacteria are representatives of more than one genus. Strain BC1 and strain ALV belong to the "low" G+C branch of Gram positive bacteria as typified by the genera *Bacillus* and *Clostridium*, whilst strain TH3 appears to be more closely affiliated with the "high" G+C group of Gram positive bacteria.

Of the moderate thermophiles described, strain BC1 has been one of the most studied bacteria (see Norris, 1989a and 1990) and was therefore chosen as the representative organism for this group of bacteria in the majority of this work. Both strain BC1 and *S. thermosulfidooxidans* exhibit a similar G+C DNA content of 50 (Harrison, 1986) and 53 (Golovacheva, 1979), respectively. Furthermore, recent comparative SDS-PAGE analysis (P. Norris and D. Clarke, pers. comm.) and DNA:DNA hybridization studies (P. Norris and J. Owen, pers. comm.) of these two organisms have indicated a 95% homology; the latter indicating a similarity at the species level.

The morphology of the moderate thermophiles varies between species and according to the growth conditions (see Norris, 1990).

These thermophiles represent the most nutritionally versatile mineral-oxidizing eubacteria, being capable of autotrophic, chemolithoheterotrophic (Norris and Barr, 1985; Marsh and Norris, 1983b) and mixotrophic (Wood and Kelly, 1983) types of nutrition. A reduced source of sulphur is required for biosynthesis during autotrophic growth by all group members except strain ALV (see Norris, 1990).

Although their maximum rates of autotrophic growth on ferrous iron are slightly slower than that of *T. ferrooxidans* (Norris, 1990), their growth yields of approximately 0.3 g dry weight per mole of ferrous iron oxidized (Wood and Kelly, 1985) are similar. These maximum

autotrophic rates can be superseded by either mixotrophic growth rates with fructose or sucrose (Wood and Kelly, 1984) or chemolithoheterotrophic growth rates with ferrous iron and yeast extract (Marsh and Norris, 1983b).

The activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), a key indicator of the Calvin cycle (see section 1.3.3), has been demonstrated during autotrophic growth of the moderate thermophiles (Wood and Kelly, 1985).

During chemolithoheterotrophic growth on yeast extract and ferrous iron, the moderate thermophiles obtain their cellular carbon from the yeast extract without significant contribution from atmospheric CO_2, and their energy from the inorganic substrate (Wood and Kelly, 1983).

Norris (1989b) observed the derepression of a 56 kDa polypeptide during autotrophic growth of strain BC1 with limiting levels of CO_2. Furthermore, this polypeptide was almost completely repressed during chemolithoheterotrophic growth with yeast extract. In light of this observation and that of Wood and Kelly (1985), this 56 kDa polypeptide was almost certainly the large subunit of RuBisCO.

These results suggest that the nutritionally versatile moderate thermophiles exert strict control over their Calvin cycle enzymes. Further examples of this type of control are common in facultative autotrophic bacteria (see Tabita, 1988).

A small number of Gram negative, "*T. thiooxidans*-resembling", sulphur-oxidizing, moderate thermophiles have been identified. One of these thermophiles, strain BC13, is capable of faster sulphur-oxidation than the Gram positive thermophiles (Norris *et al.*, 1986).

1.2.3 The extreme thermophiles

This group of archaebacteria, as typified by species of *Sulfolobus*, are morphologically similar, despite a diversity in their taxonomy and iron- and sulphur-oxidizing capacities (see Norris, 1990). The use of

mainly uncharacterized strains in mineral oxidation studies has made comparisons of the extreme thermophiles difficult.

The optimum growth temperature of *Sulfolobus* strains BC and LM has been determined at around 70°C, whilst *Metallosphaera sedula* with a DNA (mole G+C) content of 45% has demonstrated activity at 85°C (see Huber *et al.*, 1986).

The specific enzymes of the Calvin cycle (see section 1.3.3) appear to be absent in *Sulfolobus* and similar mineral-oxidizing archaebacteria (Brierley and Brierley, 1986). Although the pathway of CO_2 assimilation has not been resolved, a reductive carboxylic acid cycle (see 1.3.2) has been implicated with the functioning of an acetyl-CoA carboxylase (Norris *et al.*, 1989).

The extreme thermophiles generally exhibit a greater tolerance towards increasing acidity than, for example, *T. ferrooxidans* (Norris, 1989a) and a significantly greater iron dissolution rate during autotrophic growth with air levels of CO_2 than either the mesophiles or the moderate thermophiles (see Fig. 1.1). However, the major restriction limiting industrial application of this group of archaebacteria is probably their inhibition or destruction by agitation in the presence of high mineral concentrations (Norris and Barr, 1988).

1.3 Carbon dioxide assimilation

A definition of autotrophs (Whittenbury and Kelly, 1977) has been proposed as:

"Autotrophs are micro-organisms which can synthesize all their cellular constituents from one or more C_1 compounds."

Since elucidation of the CO_2 fixation pathway in *Chlorella* by Calvin (1962) two further pathways have now been identified that enable a

diverse spectrum of organisms to reduce CO_2 to organic carbon. These are the reductive acetyl-CoA pathway and the reductive tricarboxylic acid pathway.

1.3.1 The acetyl-CoA pathway

Acetogens, methanogens and many sulphate-reducing bacteria (Codd and Kuenen, 1987) are capable of satisfying their total cellular carbon requirements from an initial fixation of CO_2 via the acetyl-CoA (Ljungdahl-Wood) pathway; for reviews see Ljungdahl, 1986; Fuchs, 1986; Jones *et al.*, 1987. The pathway produces acetyl-CoA from two molecules of CO_2. Although the carboxyl carbon of the acetyl-CoA formed is consistently derived either from the reduction of CO_2 to carbon monoxide (CO) by carbon monoxide dehydrogenase, or from the direct binding of CO to acetyl-CoA synthase, differences do occur with the nature of the methyl carbon transporting compound. In methanogens, methyltetrahydromethanopterin (an intermediate in the reduction of CO_2 to methane) is believed to transport the methyl carbon to acetyl-CoA, whilst in *Clostridium*, a corrinoid protein is involved (Ladapo and Whitman, 1990).

For some time scepticism has surrounded the role that this pathway played within the methanogenic archaebacteria; mainly through the detection of extremely low levels of the pathway's key enzyme, carbon monoxide dehydrogenase (Shieh and Whitman, 1988). However, recent work with acetate auxotrophs of *Methanococcus maripaludis* (Ladapo and Whitman, 1990) has provided direct genetic evidence for the functioning of this pathway in these organisms.

1.3.2 The reductive tricarboxylic acid pathway

A reductive trioarboxylic acid (TCA) cycle of CO_2 assimilation has been demonstrated in a wide variety of anaerobes, including the phototrophic green sulphur bacterium *Chlorobium limicola* (Fuchs *et*

al., 1980a and b), the sulphate-reducing bacterium *Desulfovibrio hydrogenophilus* (Schauder *et al.*, 1987), and within the archaebacteria, *Thermoproteus neutrophilus* (Schafer *et al.*, 1989) and *Sulfolobus brierleyi* (see Tabita, 1988).

However, only extremely low activities for the unique carboxylation reactions (the ferredoxin-dependent pyruvate and α-ketoglutarate synthases) have been detected in some of these organisms (see Tabita, 1988). It is therefore possible that the reductive TCA cycle may not function as a major CO_2 fixation pathway, but may alternatively play a supportive role in CO_2 fixation. Further evidence for this has been provided by the cyanobacteria in which some species, although utilizing the Calvin cycle as their primary CO_2 fixation pathway, have been shown to fix as much as 20% of their total fixed carbon into C_4 acids (primarily aspartate and malate) via phosphoenolpyruvate (PEP) carboxylase (Coleman and Colman, 1981; Owttrim and Colman, 1987). However, following insertional inactivation of the gene encoding PEP carboxylase in *Synechococcus* PCC 7942 (Luinenburg and Coleman, 1990), the organism demonstrated an obligate requirement for this enzyme since it was no longer viable. Furthermore, the addition of exogenous TCA cycle intermediates and amino acids could not bestow upon this organism the ability to grow. This evidence therefore suggests that PEP carboxylase may perform more than just an anapleurotic role in *Synechococcus*.

Although many autotrophic species utilize these alternative routes of CO_2 fixation, it is clear that the Calvin cycle is by far the major CO_2 assimilatory pathway in the biosphere (Tabita, 1988).

1.3.3 The Calvin cycle

A wide spectrum of organisms are capable of CO_2 fixation via the Calvin cycle. Amongst these are the aerobic chemolithotrophs, virtually

all of the photosynthetic bacteria, various *Pseudomonas* and *Rhizobium* species, certain methylotrophs, eukaryotic algae and green plants.

The Calvin cycle (fig. 1.2) is highly conserved in prokaryotes and eukaryotes (with the exception of the pyridine nucleotide specificity of glyceraldehyde-3-phosphate dehydrogenase) and involves thirteen reactions with the net production of a C_3 compound (equation 1).

$$3CO_2 + 6NAD(P)H + 6H^+ \longrightarrow \text{glyceraldehyde-3-phosphate} + 6NAD(P) + 9ADP + 8P_i \quad (1)$$
$$+ 9ATP$$

Whilst nine of these reactions are common to the central carbon metabolism of heterotrophs, only the four reactions catalysed by phosphoribulokinase (PRK), ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), fructose-1,5-bisphosphate/sedoheptulose-1,7-bisphosphate aldolase (ALD) and fructose-1,6-bisphosphate/sedoheptulose-1,7-bisphosphate (FBPase/SBPase) are specific for carbon flow within the Calvin cycle.

Reviews of this pathway of CO_2 fixation are extensive (Dijkhuizen and Harder, 1984; McFadden, 1989; Bowien, 1989; Tabita, 1988) and almost exclusively focus on the two enzymes RuBisCO and PRK.

Ribulose-1,5-bisphosphate carboxylase/oxygenase

RuBisCO is undoubtedly the most abundant protein on earth (Ellis, 1979) and has been the subject of several detailed reviews (e.g. Miziorko, 1983; Andrews and Lorimer, 1987; Tabita, 1988; McFadden, 1980). The enzyme has a low turn-over number (1000 to 2000 mol CO_2 fixed/ mol of enzyme per min; Tabita, 1988) which may be reduced further through its competitive oxygenase activity (see fig. 1.3).

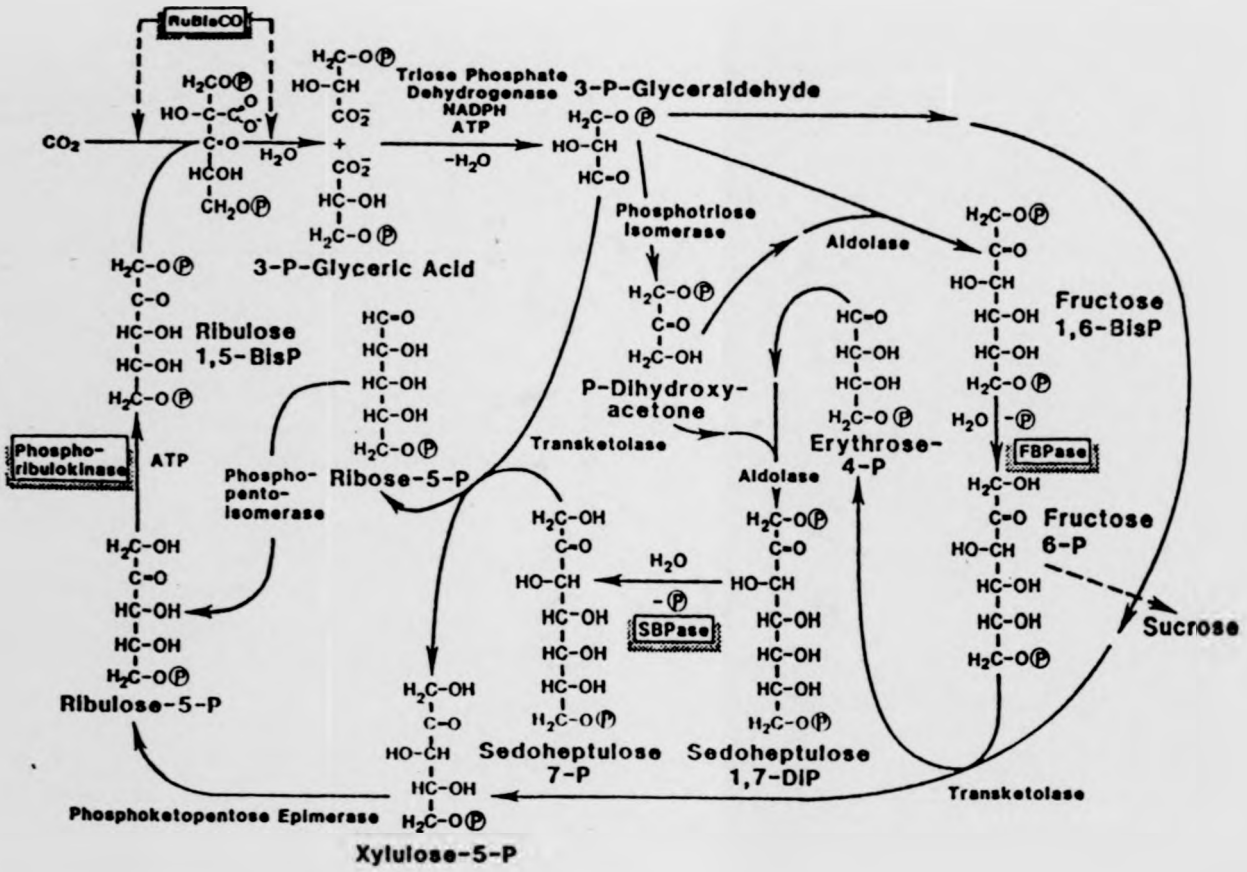
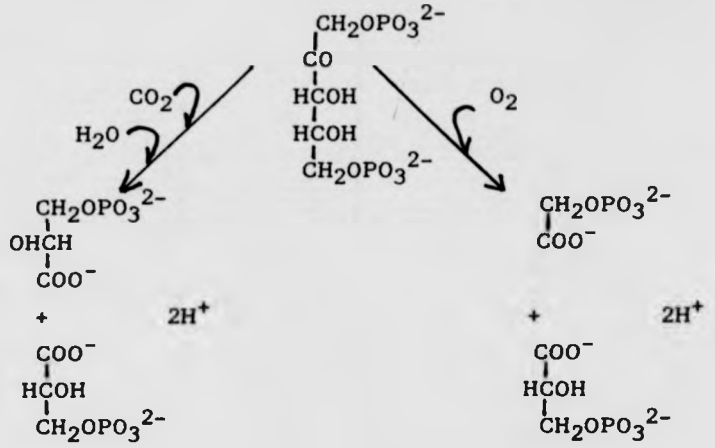


Figure 1.2 The Calvin cycle. The following enzymes, which function uniquely in the cycle, are highlighted by shading: RuBisCO (ribulose bisphosphate carboxylase/oxygenase), phosphoribulokinase, and SBPase (sedoheptulose-1,7-bisphosphatase). In addition, FBPase (fructose-1,6-bisphosphatase), is shaded to stress the fact that in some prokaryotes this activity and SBPase may occur together in a bifunctional enzyme. From McFadden, 1989.



CARBOXYLASE

2 molecules of phosphoglycerate

OXYGENASE

1 molecule phosphoglycerate
+
1 molecule phosphoglycolate

Fig. 1.3. The carboxylation/oxygenation of ribulose-1,5-bisphosphate (from Andrews and Lorimer, 1987).

Activation of both the carboxylase and oxygenase activity of RuBisCO is a two step process involving the initial and rate limiting binding of CO_2 or O_2, followed by its rapid association with Mg^{2+} (Andrews and Lorimer, 1987).

The prevalent structure of RuBisCO is a native protein of approximately 540 kDa, composed of eight large subunits (55 kDa) and eight small subunits (15 kDa). Although this L_8S_8 structure is highly conserved throughout both the prokaryota and eukaryota, good evidence exists for the presence of variations on this basic theme in some photosynthetic bacteria.

The RuBisCO isolated from *Rhodospirillum rubrum* is devoid of small subunits (Tabita and McFadden, 1974a and b; Schloss *et al.*, 1979) whilst *Rhodobacter sphaeroides*, in addition to the L_8S_8 (form I) type, possesses a L_n (form II) type of RuBisCO of uncertain aggregation state (Gibson and Tabita, 1977a and 1985). The two molecular forms in *R. sphaeroides* are distinct both immunologically (Gibson and Tabita, 1977a and b) and by peptide mapping (Gibson and Tabita, 1985). It was originally thought that the form I and form II type RuBisCOs of *R. sphaeroides* were independently regulated (Jouanneau and Tabita, 1986), however, more recent evidence (Hallenbeck *et al.*, 1990b) suggests that this may not be the case. The presence of type II RuBisCOs has also been reported in *Nitrobacter hamburgensis* (Harris *et al.*, 1988), various *Rhodospirillaceae* (Taylor and Dow, 1980; Sani, 1985; Shively *et al.*, 1984; Tabita, 1988), *Chromatium vinosum* (Torres-Ruiz and McFadden, 1987a) and certain thiobacilli (Shively *et al.*, 1989). However, in some of the above cases, evidence has been contradictory or inconclusive and must be viewed with caution (see Tabita, 1988).

The genes encoding the large and small RuBisCO subunits of *Thiobacillus ferrooxidans* have recently been cloned and sequenced (Kusano *et al.*, 1991a; Pulgar *et al.*, 1991). Although two sets of RuBisCO structural genes exist in this organism (Kusano *et al.*, 1991b) the evidence suggests that both code for the typical L_8S_8 (form I) type RuBisCO. The nucleotide and deduced amino acid sequences for this enzyme in *T. ferrooxidans* showed greater homology with RuBisCO from the photosynthetic bacterium *Chromatium vinosum* than to that from the chemoautotroph *Alcaligenes eutrophus*.

Holden (1991) has sequenced residues 73 to 200 of the gene encoding the RuBisCO large subunit (lsu) from both the moderate thermophile NMW-6 (closely related to strain BC1) and *T. ferrooxidans* strain TF1-35 (table 1.1).

The nucleotide and deduced amino acid sequences for NMW-6 exhibited closest homology with the hydrogen bacterium *Alcaligenes eutrophus* whilst the sequence data for *T. ferrooxidans* displayed greatest similarity with RuBisCO from *Chromatium vinosum*, *Spinacea oleracea* and *Synechococcus*. In contrast, no significant homology was observed between the form II type RubisCO of *R. rubrum* and either NMW-6 or *T. ferrooxidans*.

Table 1.1

A comparison of the RuBisCO lsu (residues 73-200) nucleotide and deduced amino acid sequence from the moderate thermo-acidophile NMW-6 with RuBisCO from various bacterial and plant origins (from Holden, 1991).

	Sequence homology (%)	
	amino acids	nucleotides
Thiobacillus ferrooxidans	64	60
Alcaligenes eutrophus	72	66
Chromatium vinosum	61	62
Synechococcus sp.	63	62
Spinacea oleracea	66	66
Rhodospirillum rubrum	25	39

T. ferrooxidans, *Chromatium*, *Synechococcus* and *Spinacea* have 17 amino acid residues completely conserved which were substituted in both NMW-6 and *A. eutrophus*. 11 of these being identical substitutions.

Although several theories have been proposed regarding the role of the RuBisCO small subunit (Andrews and Ballment, 1983; Torres-Ruiz and McFadden, 1987b), its exact function is still uncertain. One characteristic of RuBisCO enzymes lacking small subunits is their low specificity factor (Jordan and Ogren, 1981); the major cause of which is a high $K_m(CO_2)$. The cyanobacteria are exceptional here in that they exhibit a very high $K_m(CO_2)$ for a form I type RuBisCO. However,

this is paralleled with a high $K_m(O_2)$ and maintains the enzyme's specificity factor within the range associated with that for a form I type RuBisCO. In contrast, form II type RuBisCOs exhibit at least a three-fold lower specificity factor (Jordan and Ogren, 1981 and 1983). Table 1.2 illustrates the higher $K_m(CO_2)$ values that form II type RuBisCOs generally display relative to most form I types.

Phosphoribulokinase (PRK)

In comparison with the very well studied RuBisCO, relatively little is known about the enzyme PRK which is responsible for regeneration of the CO_2 acceptor, ribulose-1,5-bisphosphate, by phosphorylation.

The prokaryotic forms isolated to date are composed of either six (Tabita, 1980), or more typically eight (see Bowien, 1989; Siebert *et al.*, 1981), identical subunits with a holoenzyme molecular weight ranging from 220 to 256 kDa. In contrast, PRK from algal or plant origin is constructed as a dimer from subunits 41 to 46 kDa (see Bowien, 1989).

In contrast to the ferredoxin/thioredoxin-type system operational in oxygenic phototrophs, the chemolithotrophs and anoxygenic phototrophic bacteria control enzyme regulation by the use of metabolic effectors (Crawford *et al.*, 1984). Furthermore, regulation at the level of enzyme activity is achieved through control of the two enzymes PRK and FBPase/ SBPase.

Owing to the high energy demand of the Calvin cycle (equation 1), one would expect intracellular compounds reflecting the cell's energy charge to function in its control. In fact AMP has been demonstrated to inhibit PRK activity in a range of bacteria (*Thiobacillus ferrooxidans, Alcaligenes eutrophus, Rhodobacter sphaeroides* and *Chromatium vinosum*), whilst no effect has been observed only in *Thiobacillus neapolitanus* and *Rhodopseudomonas acidophilus* (see

Table 1.2. Ribulose-1,5-bisphosphate carboxylase/oxygenase K_m variations.

		K_m for:	CO_2 (μM)	RUBP (μM)	Source
Plants	terrestrial (46 genera)	C3	19±3	36±20 (mean)	1
		C4	31±2		
	aquatic (10 genera)		43±9		
Synechococcus sp.			250	45	2
Anabaena sp.			293	21	
Thiobacillus versutus			53		3
Thiobacillus neapolitanus			19	92	4
Thiobacillus ferrooxidans			28	80	5
Rhodobacter sphaeroides					
form I			36	NG	6
form II			80	NG	
Rhodopseudomonas blastica					
form I			40	48	7
form II			102	51	
Rhodospirillum rubrum			89	NG	6

1 Yeoh *et al.*, 1981.
2 Miziorko and Lorimer, 1983.
3 Charles and White, 1976.
4 Snead and Shively, 1978.
5 Holuigue *et al.*, 1987.
6 Jordan and Ogren, 1981.
7 Sani, 1985.
NG not given.

Bowien, 1989). Furthermore, NADH (NAD(P)H in plants and cyanobacteria) specifically activates PRK in all hydrogen bacteria, *Nitrobacter winogradski* and the non-sulphur bacteria; exceptions showing no activation being *Thiobacillus neapolitanus* and *Chromatium vinosum* (see Bowien, 1989).

A further mechanism (at present unknown) operates in *Alcaligenes eutrophus* (Leadbeater and Bowien, 1984). Following a switch from autotrophic to either heterotrophic or mixotrophic growth (especially on pyruvate), PRK activity is repressed and the CO_2 fixation rate reduced. This inhibition is then reversed following organic carbon depletion or a switch back to autotrophic conditions.

In addition to AMP, phosphoenolpyruvate has demonstrated inhibition of PRK activity in several organisms (see Bowien, 1989). This C_3 compound may function as a feedback inhibitor as well as an indirect indicator of the cell's energy state.

Of the data available regarding FBPase/ SBPase regulation, the evidence suggests that in chemolithotrophic bacteria the enzyme/s are not affected by AMP whilst those from some non-sulphur/purple bacteria show inhibition by ATP (see Bowien, 1989).

Finally, a characteristic of activated RuBisCO (though only those types possessing small subunits) is its *in vitro* inactivation by 6-phosphogluconate (Bowien, 1977; Sani, 1985; Tabita, 1988). However, it is uncertain how significant a role this compound plays *in vivo*.

In view of the Calvin cycle's high energy requirements it is not surprising that bacteria with versatile nutritional capabilities (i.e. facultative chemolithotrophs) exert a much stricter control over the synthesis of Calvin cycle enzymes than do obligate chemolithotrophs. For example, the presence of organic compounds has no effect on the synthesis of Calvin cycle enzymes in the specialist autotroph *Thiobacillus neapolitanus* whilst mixtures of such organic compounds

with formate generally result in the complete repression of Calvin cycle enzymes in the nutritionally versatile *Pseudomonas oxalaticus* (see Dijkhuizen and Harder, 1984). Furthermore, growth of *Pseudomonas oxalaticus* on mixtures of glyoxylate or fructose in addition to formate allowed the utilization of both substrates with the concomitant expression of the heterotrophic and autotrophic carbon assimilatory pathways (Dijkhuijzen and Harder, 1984).

Although most data refer to RuBisCO activity alone, the derepression of Calvin cycle enzymes during autotrophic growth (especially under limiting CO_2) has been well documented in many bacteria, including the thiobacilli (Smith *et al.*, 1980; McCarthy and Charles, 1974; Beudeker *et al.*, 1980), hydrogen bacteria (Bowien and Schlegel, 1981) and the *Rhodospirillaceae* (see Tabita, 1988).

1.3.4 Genetic organization in carbon dioxide fixation

Significant advances in elucidating the genetics of CO_2-fixing (*cfx*) enzyme systems have been made in the last decade and have mainly focussed on *Alcaligenes eutrophus, Rhodobacter sphaeroides* and *Xanthobacter* sp. (for reviews see Tabita, 1988; Bowien, 1989; Tabita *et al.*, 1993; Bowien, *et al.*, 1993).

In *A. eutrophus* the genome contains two identically organized clusters of twelve structural *cfx* genes which form two large operons (Bowien *et al.*, 1993). One of these clusters is located on the chromosome and the other on the pHG1 megaplasmid, each giving rise to the formation of isoenzymes of unknown significance (Bowien *et al.*, 1993). However, the presence of one operon alone is sufficient to allow autotrophic growth, as demonstrated by the type strain which lacks the plasmid genes (Bowien *et al.*, 1993). Each operon encodes 8 of the 10 Calvin cycle enzymes, with the RuBisCO genes (*rbcLS*) as initial and the fructose-1,6-bisphosphate/sedoheptulose-1,7-bisphosphate aldolase gene (*cfxA*) as terminal genes (Bowien *et al.*, 1993). Only the

genes encoding for the triose and pentose phosphate isomerases are absent from the *cfx* operons. Three further genes (*cfxX*, *cfxY* and *cfxZ*) code for proteins of unknown function (Bowien *et al.*, 1993). Both of the *cfx* operons (approximately 13 kb) are controlled by the chromosomally-encoded *cfxR* gene which encodes a transcriptional activator (Bowien, 1989). Mutants defective in this gene fail to grow autotrophically since their *cfx* operons can no longer be expressed (Bowien *et al.*, 1993). The promoter regions of *cfxR* and the *cfx* operon overlap back-to-back and are transcribed in opposite directions (Bowien *et al.*, 1993). In contrast to the *cfx* operons which are repressed to varying extents in heterotrophically grown cells, *cfxR* is constitutively expressed at low levels (Bowien *et al.*, 1993). Sequence comparisons have identified *cfxR* as a member of the *LysR* family of bacterial transcriptional regulators, mostly activators (Windhovel and Bowien, 1991). A similar activator has recently been identified upstream of (*rbcLS*) in *Chromatium vinosum* (Viale *et al.*, 1991) and in *X. flavus* H4-14 (Meijer *et al.*, 1991).

Rhodobacter sphaeroides possesses two Calvin cycle structural gene clusters, although both are chromosomally encoded (Tabita *et al.*, 1990). The A operon (containing the form I RuBisCO gene) is located on the large chromosome whilst the B operon (containing the form II RuBisCO gene) is present on the small chromosome (Tabita *et al.*, 1990). Both *cfx* operons are regulated by the product of a single, divergently transcribed, *lysR*-type transcriptional activator gene (*cfxR*) located upstream of the A (form I) operon (Tabita *et al.*, 1993).

Genes encoding fructose-1,6-bisphosphatase (*fbp*), PRK (*prk*) and aldolase (*fba*) are located upstream from the *rbcLrbcS* (form I RuBisCO) and *rbpL* (form II RuBisCO) genes of the A and B operons, respectively (see Tabita *et al.*, 1993). Each form of PRK and RuBisCO have been shown to play a distinct role in CO_2 metabolism and cellular redox maintenance when cells are exposed to excess or

limiting CO_2 concentrations, the regulation of which is at the level of transcription (Hallenbeck *et al.*, 1990b). The B operon also contains two open reading frames (ORFs) between *prkB* and *fbaB* which encode for transketolase (*tkl*) and glyceraldehyde-phosphate dehydrogenase (*gapB*). This represents the first transketolase gene sequenced from any bacterium (Chen *et al.*, 1991). Complementation studies with mutants incapable of derepressing RuBisCO synthesis (AUT^- strains) and those incapable of growing with CO_2 as the electron acceptor (CEA^- strains) have allowed the identification of at least three separate regulatory gene loci. However, to date, sequencing has not allowed identification of a protein that would provide a clue as to its role in autotrophic CO_2 fixation (see Weaver and Tabita, 1985; Rainey and Tabita, 1989).

Several variations on the above genetic organization of *cfx* operons in *R. sphaeroides* and their regulation by a single transcriptional activator have been identified in other purple nonsulphur bacteria (see Tabita *et al.*, 1993).

Recent studies with *Rhodospirillum rubrum* indicate that the promoter for *rbpL* (form II RuBisCO) is immediately upstream of this gene and that *cfxR*, which is located 5' to *rbpL*, is transcribed in the same direction (Leustek *et al.*, 1988). The *fbp*, *prk* and *tkl* genes in this bacterium are located upstream of *cfxR* and *rbpL* and are divergently transcribed (Tabita *et al.*, 1993).

To date, no other Calvin cycle structural genes have been identified in close association with the RuBisCO genes of *Chromatium*, although a divergently transcribed *cfxR*-like gene has been shown to control transcription of at least one of the *rbcLrbcS* pairs (Viale *et al.*, 1991).

In *Rhodobacter capsulatus* the form II operon is organized very similarly to that of *R. sphaeroides*, however, the *rbcLrbcS* genes of the form I operon do not appear to be closely associated with any other Calvin cycle structural genes (see Tabita *et al.*, 1993).

Finally, data provided by Meijer *et al.* (1991) *Xanthobacter flavus* have allowed identification of the genes encoding fructosebisphosphatase and PRK. These genes are located downstream of *rbcS* and are co-transcribed in the same direction as *rbcLS*. *CfxR* is located upstream of *rbcLS* and is divergently transcribed.

1.4 Inorganic carbon (C_i) transport

In contrast to terrestrial plants, aquatic autotrophs (especially in freshwater) are faced with a unique problem regarding CO_2 acquisition and its availability to the key enzyme RuBisCO. This has arisen from a 10^4-fold lower diffusion coefficient for CO_2 in aqueous medium than in air (Badger, 1987) and is further complicated by variations in dissolved inorganic carbon (DIC) experienced by these organisms resulting from pH, turbulence and cell concentration perturbations.

The physical chemistry of CO_2 is such that, in water, the carbon atom is susceptible to nucleophilic attack by both H_2O (equation 2) and OH^- ions (equation 3):

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \rightleftharpoons CO_3^{2-} + 2H^+ \qquad (2)$$

$$CO_2 + OH^- \rightleftharpoons HCO_3^- \rightleftharpoons CO_3^{2-} + 2H^+ \qquad (3)$$

The rate limiting step in both equations is the uncatalysed hydration of CO_2 to HCO_3^-: $t_{\frac{1}{2}}$ = 8 seconds at 30°C, pH 8.0 (Miller *et al.*, 1988 and Price and Badger, 1989c).

From equations 2 and 3 it is clear that under more alkaline conditions there will be greater concentrations of HCO_3^- and CO_3^{2-} at equilibrium. Conversely, at an acidic pH the dominant inorganic carbon species will be CO_2. Furthermore, for each pH unit increase above pH

7, there will be a ten-fold rise in the total dissolved inorganic carbon concentration (Miller, 1990). However, it should be noted that the CO_2 concentration for an open system will remain constant, irrespective of pH.

In order to combat a low environmental DIC concentration many aquatic organisms have evolved C_i concentrating mechanisms (CCMs). These mechanisms range from the well documented energy dependent transport systems in cyanobacteria (see Miller, 1990), to those that do not involve the involvement of active transmembrane fluxes of CO_2 or HCO_3^- and include the operation of acid and alkaline surface zonations, crassulacean acid metabolism (CAM)-like metabolisms and C_4-like metabolisms (see Raven, 1991).

The above transport systems enable organisms to generate a high intracellular C_i concentration and result in a high whole cell affinity for CO_2, a reduced RuBisCO oxygenase activity, and a low CO_2 compensation point. For example, although the RuBisCO from cyanobacteria exhibits a low affinity for CO_2 (in the presence of oxygen its effective $K_m(CO_2)$ is 600 µM; Pierce and Omata, 1988), low C_i acclimated *Synechococcus* cells are capable of half of their maximal growth rate ($K_\frac{1}{2}$) under total C_i concentrations of 10 to 15 µM which correspond to CO_2 concentrations of 5.6 to 8.4 nM (Miller *et al.*, 1984a).

Several studies have demonstrated that cyanobacteria and algae are capable of utilizing both CO_2 (usually constitutively) and HCO_3^- (usually inducibly in the presence of mM concentrations of sodium ions (Na^+); see Miller, 1990) as substrates for CCMs. However, in the case of non-aerated *Synechococcus* cultures, HCO_3^- transport is not Na^+-dependent (Espie and Canvin, 1987).

It remains uncertain whether the simultaneous uptake of CO_2 and HCO_3^- represents the activity of one dual purpose transport system or the action of two separate transporters (Espie *et al.*, 1988a;

Sultmeyer *et al.*, 1989; Price and Badger, 1989c; Kaplan *et al.*, 1991). Attempts to distinguish between a dual purpose transporter and two separate systems using the C_i uptake inhibitor carbon oxysulphide have been contradictory (Badger and Price, 1990; Miller *et al.*, 1989; Miller *et al.*, 1990). However, ethoxyzolamide inhibition studies by Price and Badger (1989c) have proposed that the dehydration of HCO_3^- and rehydration of CO_2 are successive steps during C_i transport, thereby suggesting a common carrier for the two C_i species. In contrast, the differential effect of Na^+ (Espie *et al.*, 1988b; Miller *et al.*, 1990) and H_2S (Espie *et al.*, 1989) on CO_2 and HCO_3^- transport and the observed low inhibition levels of CO_2 transport by HCO_3^-, about 6%, over a range of $H^{13}CO_3^-$ concentrations and transport rates (Espie *et al.*, 1991b) have suggested the functioning of two separate systems.

Two dual carrier mechanisms for CO_2 and HCO_3^- transport have been proposed in cyanobacteria (see Miller, 1990).

The first model involves a "HCO_3^- porter" as the translocating element in the complex (Volokita *et al.*, 1984) and requires CO_2, during active CO_2 transport, to be converted to HCO_3^- by a "carbonic anhydrase (CA)-like moiety" in the membrane prior to translocation.

A second model proposed by Price and Badger (1989c) differs from the model of Volokita *et al.* (1984) in that the primary C_i pump transports CO_2, not HCO_3^-, from the external medium to the interior of the cell. Consequently, the front-end "CA-like moiety" converts HCO_3^- to CO_2 during HCO_3^- transport which is then passed on to the "CO_2 porter".

Several single transporter mechanisms have been proposed for CO_2 transport in cyanobacteria (see Miller, 1990).

All of these models involve a "CA-like moiety" which may be an integral membrane protein, possibly even the "CO_2 porter" itself.

Alternatively it may be peripherally bound to the cytoplasmic side of the membrane. Evidence for the direct involvement of such CA activity has been provided through ethoxyzolamide studies by Price and Badger (1989c and d) in which this potent CA inhibitor blocked CO_2 transport by both high and low C_i grown *Synechococcus cells* without affecting intracellular CA activity. Similar inhibition of carbonyl sulphide transport, a structural analogue of CO_2, has been observed in cyanobacteria with ethoxyzolamide and H_2S/ HS^- (Miller *et al.*, 1989).

Further studies by Espie *et al.* (see Miller, 1990) have indicated that inhibition of CO_2 transport by *Synechococcus* was greater with the more lipid soluble H_2S than with HS^-. This may have suggested that the "CA-like moiety" was in fact the "CO_2 porter" itself or at least an integrally bound membrane protein.

However, it remains uncertain whether CO_2 transport is driven directly by the expenditure of energy, i.e. "primary", or whether it is coupled to an active transport system involving the movement of an ion along an electrochemical gradient, i.e. "secondary" (Miller, 1990).

Numerous single transporter mechanisms have been postulated for HCO_3^- transport in cyanobacteria; the most noteworthy of which have been described by Miller (1990).

One common feature of HCO_3^- transport by aerated cyanobacterial cells is their requirement for mM concentrations of Na^+. Although this Na^+ requirement may be pH dependent (see Miller, 1990), it cannot be replaced by either potassium, lithium or caesium ions (Kaplan *et al.*, 1984; Miller *et al.*, 1984b). At a pH lower than 7 it is uncertain whether the negation for Na^+ is a result of the operation of a H^+/HCO_3^- symport (Abe *et al.*, 1987) or an increase in the CO_2 transport rate (see Espie *et al.*, 1988b).

Should primary electrogenic transport of HCO_3^- take place in cyanobacteria then there would have to be either concomitant uptake of a cation or efflux of an anion. The electrophoretic uptake of Na^+ could fulfil this role and would also account for the Na^+-dependence of HCO_3^- transport in air-grown cyanobacteria (Miller, 1990).

An alternative model is that of electroneutral transport. However, should the exchange of HCO_3^- and OH^- drive the influx of HCO_3^-, it is likely that direct coupling to the expenditure of metabolic energy would be necessary (Miller, 1990). Although this mechanism is unlikely to operate in Na^+-dependent transport of HCO_3^- (no efflux of OH^- was observed by air-grown *Anabaena* cells during HCO_3^- transport; Kaplan, 1981), it cannot be precluded in those cells demonstrating no specific ionic requirement for HCO_3^- transport i.e. those capable of Na^+-independent transport of HCO_3^- (Espie and Canvin, 1987).

The final model proposed involves the secondary transport of HCO_3^- and would necessitate a symport stoichiometry of either almost 3 protons to 1 HCO_3^- or more than 2 Na^+ to 1 HCO_3^- (Miller, 1990).

This mechanism seems unlikely to operate in cyanobacteria for two reasons:

Firstly, should such a system exist it would be in itself electrogenic. However, no transient decrease in membrane potential was observed in air-grown *Synechococcus* cells during HCO_3^- transport (Kaplan *et al.*, 1982).

Secondly, as a result of the stoichiometry involved in the proposed HCO_3^- secondary transport mechanisms, the rate of HCO_3^- uptake would be dependent on the membrane potential. However, transient membrane depolarization by potassium addition has been shown to have no effect on the transport rate of HCO_3^- in air-grown *Anabaena* cells (see Miller, 1990).

Although the potential mechanisms for C_i acquisition by active CCMs are diverse (see Miller, 1990), the four following features are consistent in all models:

i) An energy-requiring, integral membrane C_i pump which may possess itself, or is at least closely associated with, a vectoral carbonic anhydrase (CA) activity. This CA activity is necessary since although both HCO_3^- and CO_2 are accepted as substrates, only HCO_3^- appears to be transported to the cytosol (Volokita *et al.*, 1984; Price and Badger, 1989d; Kaplan *et al.*, 1991).

ii) An associated energy supply, since CCMs require activation/ energization (Miller *et al.*, 1991; Espie *et al.*, 1991a). The *ictA* gene product has been implicated here (Ogawa, 1991).

iii) A barrier to limit C_i diffusion out of the cell, essential in order to maintain elevated intracellular CO_2 levels. The carboxysome has been strongly implicated to function here (see Reinhold *et al.*, 1991; Price and Badger, 1991; Yu *et al.*, 1992).

iv) CA activity in close proximity to the sites of carboxylation to ensure equilibrium between the C_i species. The uncatalysed formation of CO_2 from internal C_i has been estimated as too slow to allow for the observed rates of CO_2 fixation (Kaplan *et al.*, 1991).

Modification of whole cell affinity for C_i following acclimation to limiting C_i (up to 1000-fold lower $K_{\frac{1}{2}}$ values) has been well documented for photoautotrophs (cyanobacteria, microalgae, macroalgae and aquatic angiosperms); see reports/reviews (Miller *et al.*, 1990; Badger, 1987; Coleman, 1991; Miller *et al.*, 1991; Sultmeyer *et al.*, 1991; Merrett, 1991).

In contrast, description of attempts to quantify high affinity C_i transport in chemolithotrophic bacteria has been essentially confined to two papers (Holthuijzen *et al.*, 1987 and Karagouni and Kelly, 1989).

The present knowledge of CCMs in both the photoautotrophic and chemolithotrophic bacteria has been summarized below.

1.4.1 Studies with cyanobacteria

Initial *in vivo* labelling studies by Omata and Ogawa (1985 and 1986) identified a 42 kDa cytoplasmic polypeptide that was induced during low C_i acclimation. This polypeptide has since been shown not to be directly involved in C_i transport (Omata *et al.*, 1990 and Schwarz *et al.*, 1988) and has been explained as a carotenoid-binding protein (Reddy *et al.*, 1989).

Several high CO_2-requiring mutants have been generated (Price and Badger, 1991) that, although capable of wild type C_i transport, are deficient in the intracellular conversion of HCO_3^- to CO_2 in close proximity to RuBisCO within the carboxysome. The biochemical and molecular analysis of these mutants (Badger *et al.*, 1991; Kaplan, 1990; Lieman-Hurwitz *et al.*, 1991) has allowed the identification of genomic regions coding for essential components of CCMs and regions involved in the acclimation process (see page 41).

Another group of high CO_2-requiring mutants have been identified (Ogawa, 1990 and Ogawa *et al.*, 1990) that possess less than 5% of the wild-type C_i transport capacity. Complementation data suggest that at least two distinct proteins are required for C_i transport. The deduced amino acid sequence for one of these proteins (which is required for both HCO_3^- and CO_2 transport) has homology with the putative chloroplast-encoded NADH dehydrogenase (Ogawa, 1991). This protein, the *ictA* gene product, could be involved as an integral component in HCO_3^- and CO_2 transport or may be required for activation/energization of the CCMs.

There has been increasing interest in the signal responsible for the induction of CCMs during low C_i acclimation (Badger, 1987; Lieman-Hurwitz *et al.*, 1991; Coleman, 1991; Kaplan *et al.*, 1991) and several CO_2-dependent promoters (containing highly homologous regions) have been detected in *Synechococcus* (Scanlan *et al.*, 1990). However, at present it is uncertain whether these promoters are regulated by CO_2 itself of by the concentration of a related metabolite. The evidence to date favours the signal as a change in metabolite levels (e.g. an increase in flux through the photorespiratory pathway; Suzuki *et al.*, 1990) as opposed to a direct detection of the external or internal DIC concentration. Recent studies by Schwarz *et al.* (1992) have identified an open reading frame immediately down stream of *rbc* encoding for the cyanobacterial equivalent of *purK*, the eubacterial gene encoding subunit II of phosphoribosyl aminoimidazole carboxylase in the purine biosynthetic pathway. Deletion or inactivation of this region resulted in *Synechococcus* mutants that were high CO_2-requiring, although their photosynthetic affinity characteristics were similar to those of the high CO_2-grown wild type and not reduced as with other similar phenotypic mutants. Furthermore, exposure of high CO_2-grown *Synechococcus* to a period of low C_i acclimation was paralleled with the induction of *purK* transcription. It was therefore suggested that the high CO_2-requiring phenotype of the mutants had resulted from a purine biosynthesis deficiency following low C_i acclimation. The possibility was also raised that cellular purine levels may be involved in the process of low C_i acclimation in cyanobacteria.

Alternatively it may be the CCMs themselves that act as C_i sensors, since a reduction of C_i flux through the constitutive CCMs would result in a decrease in energy consumption by the transporters. This alteration in energy requirement or a change in the redox status of the transporter proteins could then induce synthesis or activation of the high affinity CCMs (Coleman, 1991).

1.4.2 Studies with microalgae

In vivo labelling has been more successful in the microalgae and has allowed the identification of several non-thylakoid membrane proteins that are synthesized during the low C_i acclimation period (Bailly and Coleman, 1988 and Spalding and Jeffrey, 1989).

A periplasmic CA has been identified in *Chlamydomonas* (Coleman and Grossman, 1984; Coleman *et al.*, 1984) which is distinct from its intracellular counterparts both immunologically and in response to inhibitors (Coleman *et al.*, 1991). Although the periplasmic CA is the major form induced during the acclimation period, the data provide evidence that at least the chloroplast CA is regulated to a degree by the external DIC concentration (Sultemeyer *et al.*, 1990).

Isolated chloroplasts from low C_i acclimated *Chlamydomonas* cells have demonstrated some capacity for C_i accumulation (Goyal and Tolbert, 1989) and a 36 kDa, chloroplastic polypeptide (Mason *et al.*, 1990) has been suggested to play a role in the CCM.

Although the analysis of high CO_2-requiring mutants has not been as successful in algae as in the cyanobacteria, several different loci appear to be involved in the functioning of algal CCMs and partial expression of these is constitutive (Suzuki and Spalding, 1989).

Finally, there has been speculation regarding the role that pyrenoids may play in C_i concentration and their possible analogous role to carboxysomes (see review by Coleman, 1991). Of interest is the accumulation of a 44 kDa polypeptide during low C_i acclimation in *Chlamydomonas* (Kuchitsu *et al.*, 1988).

1.4.3 Studies with chemolithotrophic bacteria

From the limited literature available, only *Thiobacillus neapolitanus* (Holthuijzen *et al.*, 1987) has been reported to possess an inducible, high affinity C_i transport system within this group of bacteria.

Holthuijzen *et al.* (1987) demonstrated that following acclimation to limiting C_i, *T. neapolitanus* cells derepressed RuBisCO synthesis five-fold and increased their whole cell affinity for C_i. This increase in C_i affinity was not simply a result of higher RuBisCO fixation rates. Furthermore, a rise in the pH from 6.0 (69% CO_2) to 7.5 (6.6% CO_2) was accompanied with a significant decrease in the C_i transport rate suggesting that this organism was only capable of transporting CO_2 and not HCO_3^-. It was further proved through the use of ionophores (valinomycin and nigericin) that the electric potential was the main driving force in C_i acquisition and that the electron transport system was implicated in its regulation.

In contrast, Karagouni and Kelly (1989) demonstrated that such an inducible system was absent from *Thiobacillus versutus* grown under carbon-limitation in a chemostat. They concluded that CO_2-fixation was rate limiting, either by C_i diffusion or by the functioning of a low affinity transport system, and was responsible for the organism's incapacity to grow autotrophically with air levels of CO_2.

1.5 Carboxysomes

Since first observations were made using electron microscopy over 20 years ago (Jensen and Bowen, 1961), uncertainty still surrounds the precise composition and function/s of carboxysomes (formerly known as polyhedral bodies).

The term carboxysome was first introduced by Shively (1973) following the discovery of RuBisCO within these organelles. Important questions have since been raised regarding the role/s that these inclusion bodies may play in relation to autotrophy. For reviews on carboxysomes see Shively (1974), Codd and Marsden (1984), Allen (1984), Codd (1988), Shively and English (1991), and Price and Badger (1991).

Although carboxysomes have only been shown to occur in those groups of autotrophic microorganisms that utilize the Calvin cycle, they are not ubiquitous within these groups, see tables 1.3 and 1.4.

To date, Golovacheva (1979) has provided the only recorded ultrastructural description of moderately thermophilic, mineral sulphide-oxidizing bacteria. Thin sections of the bacterium *Sulfobacillus thermosulfidooxidans* (closely related to strain BC1) failed to reveal the presence of any polyhedral bodies. However, it should be noted that in this report *S. thermosulfidooxidans* was grown chemolithoheterotrophically and that under such growth conditions the formation of carboxysomes would not have been favoured.

Carboxysomes have been shown to vary considerably in number per cell, even within a given genus: from 1 to 60 for *Thiobacillus* (Shively *et al.*, 1973; Beudeker *et al.*, 1980; Shively *et al.*, 1970), and from 1 to 200 for *Nitrobacter* (Wullenweber *et al.*, 1977; Watson and Waterbury, 1971). Furthermore, there appears to be no definite correlation between the presence of carboxysomes and the versatility of carbon nutrition within the chemolithotrophic bacteria (see Codd, 1988). The occurrence of carboxysomes within the genus *Thiobacillus* does however show good agreement when compared with the classification system of Katayama-Fujimura based on nutritional and physiological features, fatty acid composition, respiratory chain ubiquinones and DNA base composition (Codd, 1988), see table 1.4. However, the exceptions of *T. denitrificans* and possibly *T. thyasiris* (Lanaras *et al.*, 1991) are noted. The latter has recently been renamed within the genus *Thiomicrospira* (P. Norris, pers. comm.).

Within the cyanobacteria, carboxysomes are a constant feature of vegetative cells (although sometimes only one or two per cell) and may also occur in akinetes. In contrast, they have not been found in the N_2-fixing heterocysts (Stewart and Codd, 1975 and Cossar *et al.*, 1985).

Table 1.3 Carboxysome distribution amongst the nitrite- and ammonia-oxidizing bacteria (from Codd, 1988).

Organism	Nutritional capability	Presence of carboxysomes
Nitrite-oxidizing bacteria		
Nitrobacter winogradski	V [a]	+
Nitrobacter agilis	V	+
Nitrobacter hamburgensis	V	+
Nitrococcus mobilis	S [b]	+
Nitrospira gracilis	S	–
Nitrospira marina	S	–
Ammonia-oxidizing bacteria		
Nitrosocystis oceanus	S	–
Nitrosospira briensis	S	–
Nitrosomonas europea	S	–
Nitrosovibrio tenuis	S	–
Nitrosococcus mobilis	S	–
Nitrosomonas sp.	n.r.[c]	+

Table 1.4 Carboxysome distribution amongst the thiobacilli, grouped according to the Katayama-Fujimura classification system (from Codd, 1988).

Organism (*Thiobacillus* genus)	Nutritional capability	Presence of carboxysomes
Group I-1		
T. versutus (A2)	V	–
T. novellus	V	–
T. sp.	V	–
Group I-2		
T. acidophilus	V	+
Group II		
T. delicatus	V	+
T. intermedius	V	+
Group III-1		
T. denitrificans	S	–
T. thioparus	S	+
Group III-2		
T. neapolitanus	S	+
Group III-3		
T. ferrooxidans	S	+

[a] Versatile ie. facultative heterotroph
[b] Specialist ie. obligate autotroph
[c] not reported

These polygonal (usually hexagonal) inclusion bodies are typically 100-120 nm in diameter (Holthuijzen *et al.*, 1986b; Shively *et al.*, 1988), although diameters of up to 500 nm for *Thiobacillus* spp. and 900 nm for cyanobacteria (see Codd, 1988) have been reported. It was because of the appearance of these regular polygons that Bock *et al.* (1974) described these organelles as "phage-like" particles and tried to invoke a lytic response.

They are surrounded by a 3-4 nm thick membrane or shell which is characteristically a single layer membrane as apparent from numerous cell-thin sections (Codd and Marsden, 1984) and freeze-etching analysis (Murphy *et al.*, 1974). Owing to the lack of effect of certain organic solvents on the carboxysomal shells of *N. agilis* (Biedermann and Westphal, 1979) and *T. neapolitanus* (Holthuijzen *et al.*, 1986c), in addition to the electron microscopy evidence, lipids appear to be absent.

The total number of polypeptides (following SDS-PAGE) reported from isolated carboxysomes has been shown to vary from 7 (Biedermann and Westphal, 1979) to 27 (Beudeker and Keunen, 1981). However, it is not clear whether these estimates represent true inter-species differences in carboxysome composition or whether they merely reflect differences in the isolation and analysis methodologies between laboratories (Codd, 1988). Despite these discrepancies, two polypeptides (in equimolar quantities) are common to all carboxysome preparations. These correspond to the large and small subunits of RuBisCO (Lanaras and Codd, 1981; Cannon and Shively, 1983; Holthuijzen *et al.*, 1986c).

Apart from RuBisCO and four glycoprotein shell components of 120, 85, 54 and 19kDa (most common) in *T. neapolitanus* (Holthuijzen *et al.*, 1986c), the identities of the remaining carboxysomal polypeptides are unknown.

The activities of Calvin cycle enzymes other than RuBisCO, but for one exception (Beudeker and Kuenen, 1981), have not been detected from isolated, intact or broken carboxysomal preparations (Cannon and Shively, 1983; Holthuijzen *et al.*, 1986c and Shively *et al.*, 1988).

Bock *et al.* (1974) raised the possibility that carboxysomes may contain DNA because of their high 260:280 nm absorption ratio of 1.2. However, with the exception of Westphal *et al.* (1979), there have been no reports of direct DNA isolation from carboxysomes, although several attempts have been made (Holthuijzen *et al.*, 1986a and see also Codd, 1988).

Various biochemical functions have been proposed for carboxysomes in terms of the properties and role of RuBisCO, the most abundant carboxysomal protein (Shively, 1974; Beudeker *et al.*, 1981; Lanaras and Codd, 1982; Codd and Marsden, 1984).

Good evidence now exists that the carboxysome may play an active role in CO_2 fixation.

In order for carboxysomes to function as active CO_2 fixation sites *in vivo*, the RuBisCO contained within must be catalytically competent and the carboxysome wall freely permeable to both the substrates and products of the RuBisCO-catalysed reaction. To date, all carboxysomal RuBisCO preparations examined have been similar to, or identical with (in quaternary structure, immunological, activation and catalytic properties), the enzyme from the cytoplasmic fraction of the cell (Badger, 1980; Lanaras and Codd, 1981; Beudeker et al., 1981; Cannon and Shively, 1983).

The question of RuBisCO activation *in vivo* has been addressed directly by Cannon *et al.* (1991) using a ^{14}C-labelled variant of the transition state analogue 2-carboxyarabinitol 1,5-bisphosphate (CABP). In this experiment equal labelling of carboxysomal and cytoplasmic

RuBisCO was observed, suggesting that in *T. neapolitanus* both forms were active and to the same degree *in vivo*.

Beudeker *et al.* (1980) noted that CO_2-limitation during the growth of *T. neapolitanus* resulted in a five-fold increase in total RuBisCO activity compared to thiosulphate limitation (CO_2-excess). Changes in both carboxysomal and total RuBisCO activities correlated directly with variations in carboxysome volume density and number per cell. Furthermore, the carboxysomal RuBisCO in CO_2-limited cells was calculated to be 4.5-times more active per unit RuBisCO protein than was its cytoplasmic counterpart (Beudeker *et al.*, 1981).

Similar control over carboxysome and RuBisCO synthesis has been reported for *T. intermedius* (Purohit *et al.*, 1976), although complete repression was observed during chemoheterotrophic growth. Shively and English (1991) examined this derepression more closely. They found that there was no significant lag phase before carboxysome formation and that RuBisCO was immediately sequestered therein.

It has been further postulated that the carboxysome structure, and integration of carbonic anhydrase activity within, functions to improve utilization of the C_i pool by the creation of a localized zone of CO_2 generation at the sites of carboxylation (Reinhold *et al.*, 1987 and 1989), see Fig. 1.4.

Until only recently there had been no direct evidence for carbonic anhydrase activity in the carboxysome, although cellular activity has been shown to increase with carboxysome number during low C_i acclimation (Badger and Price, 1989 and Turpin *et al.*, 1984) and in some cases much of this activity has been associated with a "heavy" fraction of cell lysate which also contained carboxysomes (Badger and Price, 1989; Badger, 1987; Kaplan, 1990). The recent work of Price *et al.* (1992) with *Synechococcus* has however clearly indicated co-purification of the majority of the cell's CA activity along with its cellular RuBisCO in a highly pelletable fraction that also contained

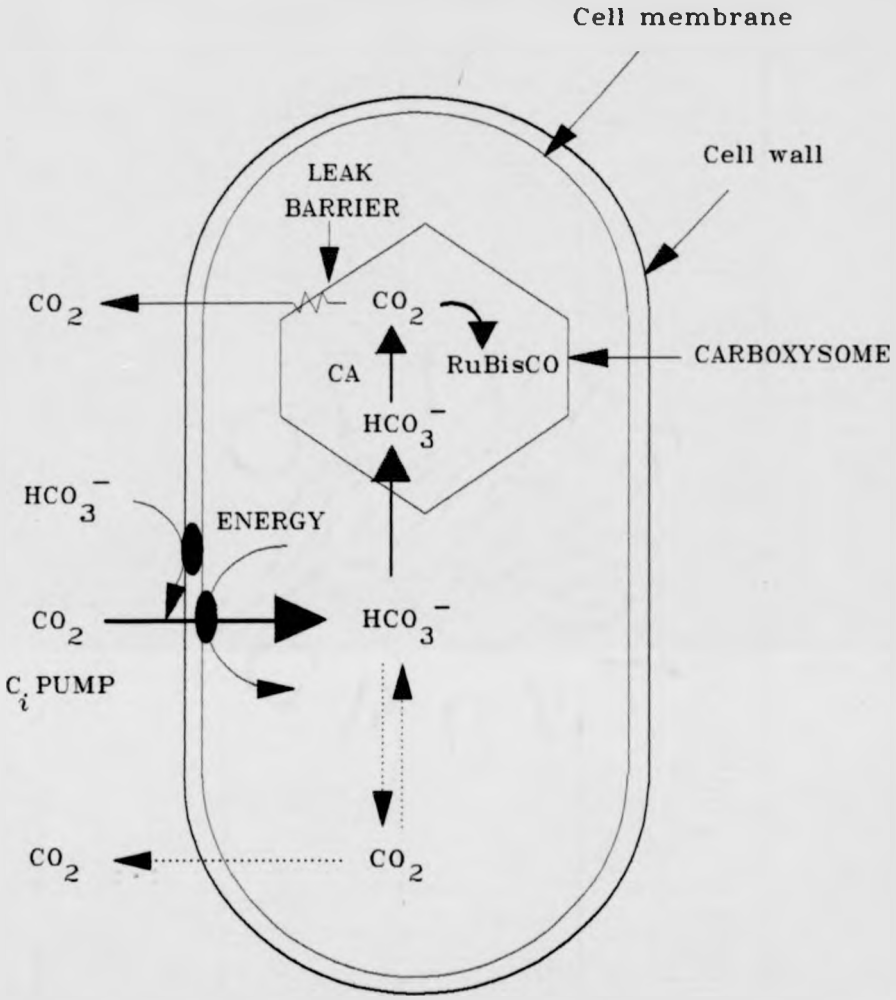


Fig. 1.4 Schematic display of the components and operation of the CCM in *Synechococcus* (from Price and Badger, 1991).

CO_2 is utilized by the C_i pump but HCO_3^- alone is delivered to the cytosol. This C_i species then passes into the carboxysome where CO_2 is generated and elevated around RuBisCO via carbonic anhydrase catalysis. An important feature of the carboxysome is its ability to limit CO_2 diffusion back into the cytosol. This property may be related to the carboxysome protein coat or the proximal arrangement of RuBisCO around CA within the carboxysome (see Reinhold *et al.*, 1991).

physically intact carboxysomes.

It had previously been thought that the lack of carboxysome-associated CA activity could be explained through the C_i flux model of Reinhold *et al.* (1989) which predicts that only extremely low levels of carboxysomal carbonic anhydrase would be required to support photosynthesis. CO_2 generation within the carboxysome may also be contributed to by the production of two protons from the RuBisCO reaction, although one of these would be consumed in the formation of 3-phosphoglycerate.

However, it now seems likely that such carboxysomal CA activity has remained elusive through a combination of the above and the use of reduced dithiothreitol in enzyme extraction buffers. This common antioxidant has recently been shown to cause inactivation of CA (Price *et al.*, 1992). When tested under optimal assay conditions, a previously isolated, high CO_2-requiring, type II mutant (see page 41) was found to display a 30-fold reduction in carboxysome-associated CA activity (Price *et al.*, 1992).

The most convincing evidence for the Reinhold *et al.* model (1989) has undoubtedly been provided by a genetic approach to elucidating the process in the cyanobacteria.

Pierce *et al.* (1989) constructed a "cyanorubrum" mutant from *Synechocystis* 6803 in which the genes encoding for RuBisCO had been substituted by those from *Rhodospirillum rubrum*. This mutant, though having a wild type C_i-concentrating capacity (Marcus *et al.*, 1992), could not produce carboxysomes and had a high CO_2-requiring phenotype.

Of further interest was the mutant's extreme sensitivity to O_2 which was much higher than could be explained by the difference in carboxylase/oxygenase ratio between the two RuBisCOs. It has been suggested that this sensitivity was due to the absence of functional carboxysomes. Another difference between the *R. rubrum* and

cyanobacterial RuBisCOs is the lack of small subunits in the former. The function of the small subunits with regard to carboxysome structure is at present unknown, although they have been implicated in formation of the carboxysomal protein coat (Kaplan, 1990; Holthuijzen *et al.*, 1986c; Lieman-Hurwitz *et al.*, 1991).

In order for the model of Reinhold *et al.* (1989) to be valid, it would be necessary for carbonic anhydrase to be located within the carboxysome whilst being absent from the cytosol and for the C_i pool to be primarily HCO_3^-. Both of these prerequisites have been implicated by the work of Price and Badger (1989a). They demonstrated that the intracellular C_i pool was rapidly equilibrated following the expression of human carbonic anhydrase in the cytosol of *Synechococcus*. This resulted in a massive leakage of CO_2 and the inability of the organism to maintain a large internal C_i pool.

Further essential evidence for this model has been provided by the generation and analysis of cyanobacterial high C_i-requiring mutants. These mutants, which are capable of C_i transport but deficient in their ability to rapidly convert HCO_3^- to CO_2 at the site of carboxylation within the carboxysome, may be grouped primarily into type I and type II classes (see Price and Badger, 1989b; Badger *et al.*, 1991). The essential phenotypic difference between these two groups is that the type I mutants possess alterations in the morphology of their carboxysomes whilst type II mutants exhibit wild type carboxysomes, although the number are often increased in the latter.

Complementation studies on these mutants suggest that regions both 3' and 5' to the genes encoding for the RuBisCO large and small subunits are responsible for assembly and functioning of the carboxysome (Friedberg *et al.*, 1989; Kaplan, 1990; Price and Badger, 1989b; Price and Badger, 1991; Lieman-Hurwitz *et al.*, 1991). Three different open reading frames (ORFs), exhibiting no significant

homology to known proteins, have been identified upstream of the *rbc* large subunit gene. Whilst insertional inactivation of two of these resulted in aberrant carboxysome formation, the inactivation of all three produced a carboxysomal-less mutant (Price and Badger, 1991). Yu *et al.* (1992) have recently isolated a 3.5 kb clone (pT2) that complements type II mutants and hybridizes to the spinach CA gene. Similar complementaion studies have been performed by Fukuzawa *et al.* (1992) on a temperature sensitive mutant of *Synechococcus*, C3P-0, that exhibits some similarity to type II mutants. The complementing 0.91 kb fragment contained a 272 amino acid-encoding ORF (*icfA*) that exhibited a deduced 22% amino acid homology to pea and spinach chloroplast CA. Insertional mutation of the *icfA* gene with a drug marker resulted in a high CO_2-requiring mutant (Fukuzawa *et al.*, 1992). It has now been confirmed that the DNA regions complementing type II and C3P-0 mutants are the same (Yu *et al.*, 1992). Finally, based on the activity of the *icfA* gene product in *Escherichia coli* it is likely that the *icfA* gene codes for the carboxysomal CA (Yu *et al.*, 1992).

Significant progress has been made towards identification of the genes involved in both C_i aquisition and carboxysome assembly through the combined techniques of recombinant DNA technology and the physiological and genetic characterization of mutants. In future, these techniques should help yield the following essential information:

i) Identification of the nature of the primary C_i pump.

ii) Clarification as to whether separate or common carrier systems exist for CO_2 and HCO_3^- transport.

iii) The precise role that carboxysomes play in C_i concentration and elevation of the CO_2 concentration.

Examination of the literature concerning the thiobacilli should allow consideration of any possible relationships between the presence or absence of carboxysomes and CCMs with the capacity of the organisms to grow autotrophically without enhanced concentrations of CO_2 in culture aeration. Table 1.5 summarizes the available information.

Table 1.5 Comparison of the autotrophic growth ability of several *Thiobacillus* sp. with CO_2 supplied as air, with the distribution of carboxysomes and the presence of an inducible, high affinity CCM.

	Thiobacillus sp.	Carboxysomes [a]	Uptake system	Growth in air
nutritionally versatile i.e. autotrophic, mixotrophic & heterotrophic	*T. versutus*	–	– [b]	– [b]
	T. acidophilus	+	?	+ [e]
	T. novellus	–	?	?
	T. intermedius	+	?	?
obligate autotrophs	*T. ferrooxidans*	+	?	+ [c]
	T. thiooxidans	+	?	+ [e]
	T. denitrificans	–	?	?
	T. thioparus	+	?	+ [e]
	T. neapolitanus	+	+ [d]	+ [d]

a Codd, 1988.
b Karagouni and Kelly, 1989.
c Norris, 1989b.
d Holthuijzen *et al.*, 1987.
e P. Norris, pers. comm.

Owing to the basic scarcity of data on CCMs for chemolithotrophic bacteria it is not possible to say whether the presence of either carboxysomes or a CCM alone is sufficient to confer growth with air levels of CO_2. However, when an organism possesses both features it has this capability, whilst if both are absent, it is devoid of such a capacity.

This study aimed to examine strain BC1, a thermophilic iron-oxidizing acidophile, in this context.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals

Unless otherwise stated, all chemicals were obtained from the following manufacturers:

British Drug Houses (BDH) Ltd., Poole, Dorset, England.

Fisons Scientific Equipment Ltd., Loughborough, England.

Sigma Chemical Co. Ltd., Poole, Dorset, England.

Radiochemicals were purchased from Amersham International plc., Amersham, Buckinghamshire, England.

Oxygen-free nitrogen was purchased from the British Oxygen Co. Ltd., London, England.

5% (v/v) carbon dioxide in air was purchased from Distillers MG Ltd., Reigate, Surrey, England.

2.2 Organisms

Two of the moderately thermophilic mineral-oxidizing bacteria utilized during this study were isolated by Marsh and Norris in (1983a); strain BC1 from a washed coal pile at Birch Coppice Colliery, Warwickshire and strain ALV from a coal spoil heap near Alvcote, Warwickshire. Strain ICC was recently isolated from an Icelandic hot spring (Norris, unpublished work).

Thiobacillus ferrooxidans (DSM 583) and *Rhodobacter sphaeroides* (NCIMB 8153) were also available in the Department of Biological Sciences, University of Warwick.

Pea (*Pisum sativum*) membrane-free extract was supplied by the Plant Biochemistry group of the Department of Biological Sciences, University of Warwick.

2.3 Growth media, conditions and assays

For *Thiobacillus ferrooxidans*, the following basic medium was used (g/L of Elgastat deionized water). Elgastat Spectrum cartridge types SC32 and SC1 were supplied by Elga Ltd., High Wycombe, Bucks., England.

	g/L
$(NH_4)_2SO_4$	0.2
$MgSO_4.7H_2O$	0.4
K_2HPO_4	0.1
KCl	0.1

The medium was adjusted to pH 1.6 with H_2SO_4 (using a Radiometer PHM 62 pH meter and glass electrode) and then autoclaved (15 psi for 15 min).

$FeSO_4.7H_2O$ (1M stock solution in water (pH 1.3), acidified with H_2SO_4) was autoclaved at 10 psi for 10 min and then added to give a final concentration of 50 mM ferrous iron.

The moderate thermophiles were also grown autotrophically in the above pH 1.6 media. However, since strain BC1 had a reduced sulphur source requirement for growth (Norris and Barr, 1985; Wood and Kelly, 1983), a 100 mM potassium tetrathionate solution (10 psi for 10 min) was added to give a final concentration of 0.5 mM tetrathionate as the reduced sulphur source.

For chemolithoheterotrophic growth, the basic medium was supplemented with a 1% (w/v) yeast extract solution (15 psi for 15 min) to give a final concentration of 0.02% (w/v).

For mixotrophic growth, the basic medium was supplemented with a 100 mM glucose solution (10 psi for 10 min) to give a final concentration of 1 mM.

These iron-oxidizing bacteria were routinely subcultured and maintained in 250 ml conical flasks containing 100 ml medium. Gas mixes of carbon dioxide in air were supplied by means of a sterile glass tube containing a cotton wool filter inserted through a sterile foam bung. The flasks were incubated in Gallenkamp orbital incubators at 30°C for *T. ferrooxidans* or 45°C for the moderate thermophiles.

For growth experiments, 1 litre reactors were used. These were fitted with a water jacket and thermostat heater (Conair Church Hill Ltd., Uxbridge, Middlesex). 5% (v/v) carbon dioxide in air was diluted with air using flow meters to deliver the required percentage of CO_2 to each reactor at a final rate of 250 ml/min. The gas supply was filtered through sterile glass wool prior to delivery to the medium via a number 2 sparger (Sartorius-AG, Göettingen, Germany). A pH of 1.6 was maintained by additions of 5% (v/v) H_2SO_4 in order to minimize ferric iron precipitation.

For larger scale biomass production 20 L glass vessels were used. These were rested on heater/stirrers and gassed at 500 ml/min. A constant temperature was maintained by means of a contact thermometer in the medium linked to a hot-plate stirrer.

In each case the inoculum, 5% (v/v), was of mid-exponential phase cells which had been serially sub-cultured at least twice under the desired experimental condition.

Iron oxidation was routinely monitored by titration of 1 ml culture aliquots (in triplicate) with indicator (1, 10-phenanthroline-ferrous sulphate complex solution) against 0.05 M ceric sulphate solution (in 5% (v/v) H_2SO_4) using a 50 ml digital burette (Brand, Germany).

Cell protein was determined by the method of Lowry *et al.* (1951). Triplicate samples of between 10 ml and 100 ml were filtered through 0.4 µm nitrocellulose discs (Sartorius-AG, Göettingen, Germany). Identical filters were also included with the protein standards to allow for an observed "quenching" effect of the protein by the filters.

Cell numbers were monitored using a haemocytometer counting chamber (10x eye piece, 40x objective lens).

R. sphaeroides was grown in continuous culture under carbon dioxide limitation on butyrate-HCO_3^- medium (Gibson and Tabita, 1977a) at a dilution rate of 0.05 h^{-1}. The culture was degassed with oxygen-free nitrogen and then illuminated at 10,000 lux during growth at 30°C.

2.4 Cell suspension preparations

Small culture volumes (up to 2 L) were harvested by centrifugation at 10,000 x g for 15 min in a fixed angle Beckman JA10 or JA20 rotor at 4°C. Larger volumes were initially harvested by continuous cross-flow filtration. A Verder 2006 pump (Antwerp, Germany) was used to drive cells across a Filtron Omega 0.3 µm open channel filter (Flowgen Instruments Ltd., Sittingbourne, Kent) until the final volume had been reduced to less than 2 L.

Following harvesting, the cells were resuspended in pH 1.6 medium in the absence of ferrous iron, or in buffer (as appropriate) and centrifuged at 500 x g for 5 min. This was to remove any ferric iron precipitates before final pelleting and washing.

When large scale biomass production necessitated cell storage for longer than 24 hours, washed pellets were drop frozen in liquid nitrogen and stored at -20°C until required.

2.5 Cell lysis

Lysozyme was used with cells that were to be exposed to denaturing PAGE (2.6). Cells were washed and resuspended in 50 mM Tris, 10 mM EDTA (pH 8.5). One tenth volume of lysozyme (5 mg/10 ml) was then added and the cell suspension incubated at 37°C for 30 min.

In the absence of lysozyme treatment, cells were resuspended in approximately 4 ml of the appropriate buffer at 0-4°C and broken by

four passes through a pre-cooled French Pressure Cell (American Instrument Co., Silver Spring, Maryland) at 20,000 psi (40,000 psi for *T. ferrooxidans*). Unbroken cells and debris were removed by centrifugation at 15,000 x g for 15 min at 4°C.

2.6 Polyacrylamide gel electrophoresis (PAGE)

2.6.1 Denaturing (PAGE)

The following stock solutions were used.

Acrylamide/bis-acrylamide solution:

0.8 g bis-acrylamide

30 g acrylamide

made up to 100 ml with distilled water.

Tris-SDS solution (pH 6.8):

30.27 g Tris-HCl

2 g SDS

made up to 1 L with distilled water.

Tris-HCl solution (pH 8.8):

90.82 g Tris-HCl

2 g SDS

made up to 1 L with distilled water.

Ammonium persulphate solution (prepared daily):

0.1 g AMPS in 10 ml distilled water.

Running buffer:

15.15 g Tris-HCl

72 g glycine

5 g SDS

made up to 5 L with distilled water.

Standard LKB (Bromma, Sweden) electrophoresis plates (internal volume 35 ml) were cleaned with methanol prior to use. A 10% (w/v) acrylamide resolving gel was then prepared using the above stock solutions.

The 10% (w/v) resolving gel contained:

10 ml acrylamide/bis-acrylamide solution

15 ml Tris-HCl (pH 8.8) solution

3.5 ml distilled water

1.5 ml AMPS solution

7.5 µl TEMED (Sigma)

The TEMED was added immediately prior to pouring. 1 ml of distilled water was layered on top of the resolving gel before polymerization. Following polymerization the overlay was poured away and the gel surface washed with distilled water. Finally, the stacking gel was poured (see below) and the sample well comb inserted immediately.

The stacking gel contained:

1 ml acrylamide/bis-acrylamide solution

5 ml Tris-HCl (pH 6.8) solution

1 ml AMPS solution

3 ml distilled water

5 µl TEMED

After polymerization, the comb was removed and the sample wells rinsed and filled with running buffer.

Samples containing up to 200 µg protein were loaded into each well and electrophoresis was performed at 40 mA (constant current) per slab gel using an LKB 2197 power unit and 2001 vertical electrophoresis unit.

The samples were prepared by mixing with an equal volume of loading buffer (see below) in eppendorf tubes. The lids were pierced with a needle and the tubes boiled in a fume hood for 3 min. Finally, samples were centrifuged to remove any debris prior to loading in a MSE microfuge (11,600 x g for 5 min).

Loading buffer solution:

1 ml distilled water

1 ml 1 M Tris-HCl (pH 6.9)

1.6 ml glycerol (1.26 g/ml)

3.2 ml 10% (w/v) SDS

0.8 ml 2-mercaptoethanol (as supplied by Sigma)

0.4 ml 0.1% (w/v) bromophenol blue

Protein standards (Pharmacia Inc., Uppsala, Sweden) used for SDS-PAGE were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soya bean trypsin inhibitor (20.1 kDa) and alpha-lactalbumin (14 kDa).

2.6.2 Non-denaturing PAGE

The following stock solutions were used for native slab gel PAGE.

High bis-acrylamide solution:

30.0 g acrylamide

1.6 g bisacrylamide

made up to 100 ml with distilled water.

Low bis-acrylamide solution:

60.0 g acrylamide

0.3 g bis-acrylamide

made up to 100 ml with distilled water.

Lower gel buffer:

36.6 g Tris-HCl (pH 8.8)

made up to 100 ml with distilled water.

Running buffer:

30.0 g Tris-HCl

144.0 g glycine

made up to 5 L with distilled water.

Electrophoresis plates were cleaned and assembled. A 4 to 30% (w/v) gradient gel was cast using the above stock solutions, as directed below.

A 30% (w/v) solution was prepared:

5.0 ml low bis-acrylamide solution

3.7 ml 75% (w/v) glycerol

1.3 ml lower gel buffer

Immediately before pouring, 50 µl 10% (w/v) AMPS and 5 µl TEMED were added.

A 4% (w/v) solution was prepared:

3.32 ml high bis-acrylamide solution

18.50 ml distilled water

3.13 ml lower gel buffer

2 ml of this 4% (w/v) solution were removed and retained for use as a "tooth-former". Immediately before pouring, 100 µl 10% (w/v) AMPS and 10 µl TEMED were added to the rest of the solution. The gradient was cast (Fig. 2.1) using a Watson-Marlow 502S peristaltic pump. Water was used to overlay the gel which was left to polymerize for at least

4 hours. The overlay was then washed off and the "tooth-former" cast by adding 50 µl 10% (w/v) AMPS and 5 µl TEMED to the 4% solution retained for this purpose. This was immediately overlayed on the gradient gel and a suitable comb inserted.

Sample preparation and loading were as in 2.6.1 with the omissions of SDS, the boiling stage and 2-mercaptoethanol. Electrophoresis was performed at 8 mA (constant current) per slab gel overnight at room temperature.

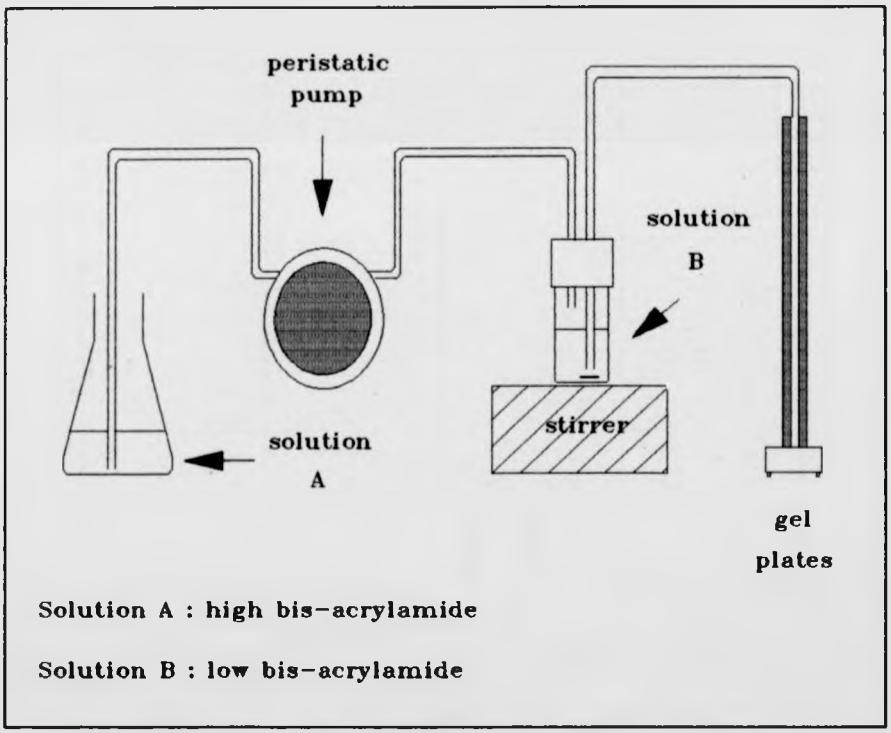


Fig. 2.1 Gradient gel-casting arrangement.

The protein markers (Pharmacia Inc., Uppsala, Sweden) used were thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa) and albumin (67 kDa).

The following stock solutions were used for native tube gel PAGE.

Acrylamide/bis-acrylamide solution:

32.0 g acrylamide

0.6 g bis-acrylamide

made up to 100 ml with distilled water.

Resolving buffer:

36.4 g Tris-HCl (pH 8.8)

made up to 100 ml with distilled water.

Ammonium persulphate solution (prepared daily):

0.14 g AMPS

made up in 100 ml distilled water.

Running buffer:

6.0 g Tris-HCl (pH 8.8)

28.3 g glycine

0.75 ml beta-mercaptoethanol

made up to 1 L with distilled water.

Glass tubes (10 cm x 0.5 cm) were cleaned, dried and marked at a length of 8 cm. They were sealed at the bottom with parafilm and a rubber band and held in a upright position by rubber grommets located in the upper buffer reservoir of the electrophoresis apparatus. A 5% (w/v) gel mix was made from the above stock solutions as below.

The 5% (w/v) gel mix contained:

6.25 ml acrylamide/bis-acrylamide solution

8.75 ml resolving buffer

20 ml AMPS solution

5 ml distilled water

20 µl TEMED

As before, the TEMED was added immediately prior to pouring. A pasteur pipette was used to fill the tubes with acrylamide solution to the mark before overlaying with water and polymerization. Finally, the gel surface was washed with running buffer and the parafilm removed from the tube base.

Samples containing up to 100 µg protein were loaded in each tube. Electrophoresis was performed at 4°C, initially at 0.25 mA per tube until the dye front had entered the tubes and then at 2.5 mA per tube until termination. The tubes were removed from their glass rods by carefully applying buffer through a syringe and needle around points of friction and finally by pressure from a Pasteur pipette bulb.

Molecular weight markers and protein samples were prepared as described for non-denaturing slab gels.

2.6.3 Gel staining

For Coomassie blue staining, gels were routinely stained overnight in:

1 g Coomassie brilliant blue R-250

400 ml methanol

100 ml glacial acetic acid

made up to 1 L with distilled water

Gels were destained by frequent volume changes in the above solution minus Coomassie blue.

For silver staining, the method of Wray *et al.* (1981) was used.

2.6.4 Western blotting

The method employed was based on Burnett (1981) with the following modifications by Greg Watson, Department of Biological Sciences, University of Warwick.

Following electrophoresis the gel was allowed to equilibrate in blotting buffer (20 mM Tris, 150 mM glycine, 20% (v/v) methanol) for about 30 min.

A sandwich was then made for the blotter as below:

A sheet of sponge 1-2 cm thick.
3 thicknesses of Whatman 3M paper.
The gel.
A sheet of nitrocellulose paper (High-bond C) just larger than the gel.
3 more thicknesses of filter paper.
Another sheet of sponge.

This was all held together by the plastic transblot holder. The sponges and all other components were wetted prior to assembly and great care was taken to avoid any air bubbles.

The sandwich was inserted into the transblot apparatus (LKB., Bromma, Sweden) with the nitrocellulose towards the anode and the tank filled with buffer. This was run at 120 mA for 18 hours.

The blot was removed and stained for protein by rinsing in Ponceau S (0.5% (w/v) in 5% (w/v) trichloroacetic acid) for 10 min. Protein

markers were marked with pencil and the blot destained in Tris buffer saline (TBS):

2.5 mM Tris-HCl (pH 8)

150 mM NaCl

The nitrocellulose was washed in 20 ml TBS + 2% (w/v) Marvel milk powder for 2 hours on a shaker.

This blocking solution was then substituted for 20 ml of fresh TBS + 2% (w/v) Marvel containing the primary antibody (5 µl) and left shaking overnight.

Anti-*Rhodobacter sphaeroides* (form I) RuBisCO antibodies were provided by Dr. J.C. Murrell, Department of Biological Sciences, University of Warwick.

Unbound primary antibody was removed by 3 x 10 min washes in 20 ml TBS + 0.1% (v/v) Tween 20.

20 ml fresh TBS + 0.1% (v/v) Tween 20 containing 60 µl goat anti-rabbit peroxidase conjugate IgG (Sigma) was added and incubated for 2 hours, shaking.

The unbound secondary antibody was removed by 2 x 10 min washes in 20 ml TBS + 0.1% (v/v) Tween 20 followed by 2 x 10 min washes in an excess volume of TBS.

Finally, the blot was stained for 5 to 20 min, washed in several volumes of distilled water and stored.

Staining solutions were:

solution A:

1.5 g NaCl + 1 ml 1 M Tris-HCl (pH 7.5)

made up to 50 ml with distilled water.

solution B:

30 mg chloronapthtol + 10 ml methanol

made up to 50 ml with distilled water.

50 µl of hydrogen peroxide was added to solution A prior to addition of solution B and used immediately.

2.6.5 Densitometer analysis and photography

Stained gels and western blots were directly analysed on a computing densitometer (Molecular Dynamics, Sevenoaks, Kent), connected to an Epsom LQ 860 printer.

Stained gels and western blots were routinely photographed using a Pentax SP500 35 mm SLR camera with Agfa-Ortho film (ASA 25).

Ilford contrast FF developer (Ilford Ltd., Mobberley, Cheshire) and Kodak universal liquid fixer (Kodak Ltd., UK) were used for the developing and fixing of both negatives and prints.

2.7 Determination of enzyme activities

Enzyme activities were essentially determined by the methods of Smith, Kelly and Wood (1980).

The following assay mixes were used.

RuBisCO assay mix (30 ml):

23 ml 0.1 M Tris-HCl (pH 8.0)

3 ml 0.25 M $MgCl_2$

112 mg $NaHCO_3$

250 µl $NaH^{14}CO_3$ (51.8 mCi/mmol)

19 mg reduced glutathione (free acid)

3.70 ml distilled water

PEP carboxylase assay mix (30 ml):

23 ml 0.1 M Tris-H_2SO_4 (pH 8.0)

3 ml 0.25 M $MgSO_4$

112 mg $NaHCO_3$

250 µl $NaH^{14}CO_3$ (51.8 mCi/mmol)

19 mg reduced glutathione

3.70 ml distilled water

Chloride ions were excluded from the PEP carboxylase assay because they were known to inhibit the activity of this enzyme in *E. coli* (Izui *et al.*, 1970).

PRK assay mix (30 ml):

23 ml 0.1 M Tris-HCl (pH 8.0)

3 ml 0.25 M $MgSO_4$

112 mg $NaHCO_3$

250 µl $NaH^{14}CO_3$ (51.8 mCi/mmol)

19 mg reduced glutathione

3.70 ml distilled water

77 mg ATP (Na salt, grade I, Sigma)

18 mg NADH (di-Na salt, grade III, Sigma)

150 mg RuBisCO (from spinach, Sigma)

Pyruvate carboxylase assay mix (30 ml):

23 ml Tris-HCl (pH 8.0)

3 ml 0.25 M $MgCl_2$

112 mg $NaHCO_3$

250 µl $NaH^{14}CO_3$ (51.8 mCi/mmol)

19 mg reduced glutathione

3.70 ml distilled water

50 mg ATP

45 mg Na glutamate

8 mg acetyl Co-A (Li-salt, Sigma)

Enzyme substrates (at their addition concentrations) for initiating reactions were:

10 mM ribulose-1,5-diphosphate (tetra-Na salt, Sigma)

60 mM phospho(enol) pyruvate (tri-Na salt hydrate, Sigma)

10 mM D-ribose-5-phosphate (di-Na salt, Sigma)

50 mM Na pyruvate

Other reagents used in enzyme assay determinations were:

Triton X-100 (diluted as required)

Cetyl trimethylammonium bromide (diluted as required)

33% (v/v) phosphoric acid (6 M)

Absorption fluid (15 ml ethanolamine: 35 ml methoxyethanol)

Optiphase " safe" scintillation fluid.

Mid-exponential cells were harvested, washed in a solution of 50 mM Tris-HCl (pH 8.0) and 10 mM EDTA to neutralize the acidic pellet. Final resuspension was to an optical density (O.D.) 440 nm of between 1.8 and 2.0 (approx. 0.6 mg dry wt/ml) .

Two ml of this suspension were added per assay tube and pelleted in a micro-angle centrifuge (Baird & Tatlock Ltd., Chadwell Heath, Essex). The supernatant was then carefully aspirated away leaving approximately 1 mg protein per tube.

For assays involving permeabilized whole cells, 0.3 ml of permeabilizing agent was added to each tube and the pellet resuspended by gentle whirlimixing. The tubes were incubated at the appropriate temperature (30°C for *T. ferrooxidans*; 45°C for strain BC1) for 15 min.

Following permeabilization, 0.9 ml of the appropriate enzyme assay mix was added and the tubes further incubated for 10 min to fully activate the test enzyme (see Wildner and Henkel, 1978; Lorimer, 1981).

When assays were performed with cell-free extracts, the permeabilization step was omitted.

After exactly 10 min, 0.3 ml of the test enzyme substrate was added to one tube. As a control, 0.3 ml of Tris-HCl (pH 8.0) was added to a parallel tube. The tubes were briefly whirlimixed before return to the water bath.

Starting from time zero, 200 µl samples were removed every 10 min and added to 200 µl 6 M phosphoric acid in 20 ml glass Packard liquid scintillation vials.

The vials were placed on a heating plate at 40°C for 1 hour in a fume hood to release any unfixed, labelled CO_2. After cooling, 10 ml of Optiphase "safe" scintillation fluid were added to each vial.

A standard count of the ^{14}C-bicarbonate mixture was performed by adding a 10 µl sample of enzyme assay mix (in triplicate) to 500 µl of absorption reagent in a scintillation vial. Ten ml scintillant were added and the counts determined at once.

Background (BG) counts were determined by counting 10 ml of scintillant alone.

Counting was routinely performed in a Beckman LS 7000 Liquid Scintillation System with an Epson FX80 dot matrix printer on-line.

Protein was determined from triplicate 1 ml samples of resuspended cells ($O.D._{440}$ 1.8 to 2.0) by the method of Lowry *et al.* (1951).

Quantification of enzyme activities was derived as follows:

i) The bicarbonate final concentration in the assay mixes was 44 mM $NaHCO_3$.

A bicarbonate specific activity, in counts per min (cpm) per nmol CO_2, was calculated as below:

$$\text{BICARBONATE SPECIFIC ACTIVITY} = \frac{[\text{ cpm (per 10 µl standard) x 100 }]}{44{,}000 \text{ nmol}}$$

$$= \frac{\text{cpm (per 10 µl standard)}}{440}$$

$$= \text{cpm/ nmol } CO_2$$

Background (BG) was subtracted to yield bicarbonate specific activity (BSA) in cpm/ nmol CO_2.

ii) BG was subtracted from all experimental counts and ^{14}C fixed was plotted against time (Fig. 2.2).

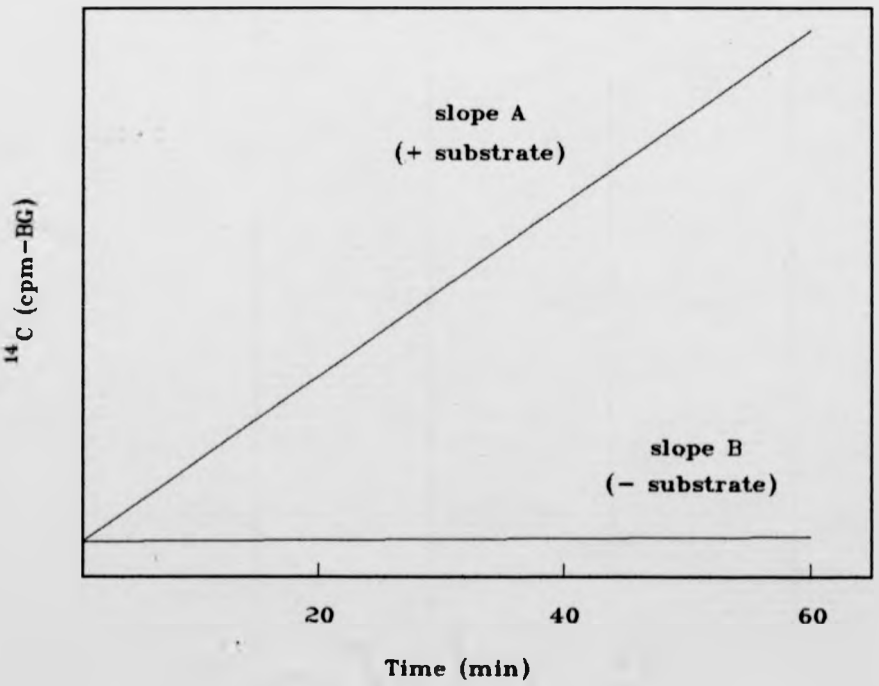


Fig. 2.2 Calculation of enzyme activity (cpm fixed/ min)

Enzymic Activity, (EA) = slope A - slope B

(cpm fixed/ min incubation) (from fig. 2.2)

Finally, the enzyme specific activity (S.A.) was calculated from:

$$\text{S.A. (nmol } CO_2 \text{ fixed/ min/ mg protein)} = \frac{\text{EA}}{\text{BSA x (protein per 200 µl sample)}}$$

2.8 Electron microscopy

A combination of the three following methods was used in sample preparation:

Kellenberger, Ryter and Sechaud (1958); Spurr (1969); Wood and Kelly (1989).

Cells were harvested, pre-fixed with 2% (v/v) glutaraldehyde in Kellenberger buffer for 2 hours and post-fixed with 2% (w/v) osmium tetraoxide in Kellenberger buffer overnight.

Excess osmium tetraoxide was removed through a repeated series of washing in Kellenberger buffer and pelleting by centrifugation. The final pellet was embedded in a minimum volume of 2% (w/v) Bacto agar. Following a drying period of 30 to 60 min the agar was sliced into 2 mm squares with a razor blade and stained with 15 (w/v) uranyl acetate in Kellenberger buffer for 2 hours.

Finally, the squares were washed twice with Kellenberger buffer before been dehydrated through a series of ethanol (v/v) gradients of 20, 40, 60, 95 and 100%. Each wash was for 30 min with an extra change in 100% ethanol.

Embedding was initiated by the addition of an equal volume of Spurr resin (Agar Aids) to the final 100% ethanol wash. The mixture was

swirled and left to stand for 30 min. Another equal quantity of embedding medium was added to the mixture and left for 30 min as before. The resin/ethanol complex was drained and a third amount of embedding medium added. The specimens, then in 100% resin, were left shaking overnight at 4°C to complete infiltration. The following day the embedding medium was replaced with fresh Spurr resin, the specimens placed at the bottom of beam capsules and the blocks polymerized by baking at 60°C for 48 hours.

Pre-sectioning was performed with a razor blade. This involved removing any rough edges from the base of the block and slicing away excess resin to expose the specimen. The block was then mounted within a clamp on a sectioning machine (Reichert, Austria) and a rhombus cut into its apex. Following this, the block was secured onto the sectioning arm and the diamond knife mounted. Water was drawn across the reservoir until the cutting edge of the knife appeared silver (due to light refraction). From the ribbon of sections floating on the reservoir, only the gold and silver (thinnest) ones were selected. These were collected on 3.05 mm OLD 100 copper grids (Agar Aids) and allowed to dry on tissue paper.

Dried sections were viewed using a Jeol JEM-100S transmission electron microscope operated at between 20 and 80 mA.

2.9 RuBisCO purification

Throughout the purification procedure fractions were tested for activity as before (see 2.7) with the following assay volume modification of 10 µl protein fraction + 100 µl RuBisCO assay mix + 20 µl ribulose-1,5-diphosphate. The reactions were performed in eppendorf tubes and terminated after 5 min. RuBisCO specific activities were determined as before, but following a reduced sampling time period of 0 to 90 seconds.

The isolation buffer (IB) and post-sucrose gradient buffer of Cook *et al.* (1991) were used.

Isolation buffer (pH 8.2):

10 mM Tris-HCl
0.5 mM EDTA
50 mM $MgCl_2$
14 mM 2-mercaptoethanol

Post-sucrose gradient buffer (pH 8.2):

10 mM Tris
0.5 mM EDTA
10 mM $NaHCO_3$
5 mM $MgCl_2$
14 mM 2-mercaptoethanol

Cells were resuspended in IB and lysed in the French Press. Centrifugation at 120,000 x g for 2 hours yielded the soluble fraction (supernatant).

The protein solution was ammonium sulphate (w/v) precipitated into four fractions (0-30%, 30-55%, 55-70% and 70-100%) according to Dawson (1974). After 30 min on ice, precipitated material was recovered by centrifugation at 30,000 x g for 30 min and dialysed against IB overnight at 4°C.

Following activity testing, the 30 to 55% dialysed ammonium sulphate fraction was ultrafiltered using a Diflow, 10 kDa cut off, PM10 membrane (Amicon Corporation, Danvers, MA, Ireland) to approximately 8 ml.

Four ml of this were layered onto each of two 35 ml sucrose step gradients (0.2 M to 0.8 M sucrose in IB, see below) and centrifuged at 90,000 x g for 18 hours in a Beckman Sw28 rotor.

The step gradient was composed of:

4 ml each step of 0.2 M to 0.6 M (inclusive)

5 ml each step of 0.7 M and 0.8 M

5 ml 30% (w/v) base layer.

After a settling period of 30 min the gradients were fractionated (1 ml) from the bottom and assayed for protein according to Bradford (1976), and RuBisCO activity.

The most active fractions were pooled and further purification was attempted by FPLC (Pharmacia, Uppsala, Sweden) using a Green-A (Amicon, Danvers, MA, Ireland) dye-ligand column. The column was eluted with a 0 to 2 M KCl gradient in post-sucrose gradient buffer. 3 ml fractions were collected and monitored for protein (absorbance 280 nm) and RuBisCO activity. Active fractions were then pooled and concentrated approximately 2 ml by ultrafiltration (as before).

2.10 RuBisCO affinity determinations

The methods employed were essentially as described by Pierce *et al.* (1982).

2.10.1 $K_m(CO_2)$ determination

The following stock solutions were used:

Assay buffer:

200 mM Bicine (pH 8.2)

1 mM dithiothreitol

0.4 mM EDTA

Other reagents:

12.5 mM ribulose-1,5-diphosphate (pH 6.5)

2 M $MgCl_2$

250 mM $NaH^{14}CO_3$ (4 µCi/µmol)

2 M HCl

The reactions were performed in 20 ml scintillation vials with all reagents except the ribulose-1,5-diphosphate and labelled bicarbonate solution added as an immediate pre-mix. Therefore each vial contained:

250 µl assay buffer

150 µl distilled water

5 µl $MgCl_2$ (2 M)

The labelled bicarbonate solution (250 mM) was diluted with 10 mM Bicine (pH 8.2) to produce the following concentrations (mM): 125, 62.5, 37.5, 20, 10 and 5. These gave final assay concentrations of (mM): 5.38, 2.69 1.61, 0.86, 0.43 and 0.22 respectively (ignoring carry-over from the activated enzyme solution).

Each bicarbonate concentration was added to an equal volume of stock ribulose-1,5-diphosphate solution as a pre-mix.

After temperature equilibration of the bicine buffer, 40 µl of the bicarbonate/ribulose-1,5-diphosphate pre-mix (20 µl of each) was added to each vial.

The reaction was then started by the addition of 20 µl enzyme solution (approximately 2 mg/ml) which had been pre-activated (at 30°C for *T. ferrooxidans* and pea; 45°C for strain BC1) for 15 min in post-sucrose gradient buffer.

Addition of 200 µl 2 M HCl sacrificed a vial at time intervals of 0, 15, 30, 45 or 60 secs and unfixed bicarbonate was released as described earlier.

As a control, 20 µl of post sucrose gradient buffer (instead of ribulose-1,5-diphosphate) was added to a series of 5 vials which were sacrificed as above.

In order to determine the bicarbonate specific activity, 5 µl samples (in triplicate) were taken from each bicarbonate concentration and added to 500 µl absorption reagent in a 20 ml vial.

The enzyme activities were calculated as follows:

Cpm (minus background) were plotted against time for each bicarbonate concentration (see fig. 3.11 for strain BC1). Following subtraction of the control rate (no ribulose-1,5-diphosphate), enzyme activities (EA) were then determined in cpm/ min of assay for each bicarbonate concentration.

The bicarbonate specific activity (BSA) for each concentration was determined in cpm/ nmol CO_2 :

$$\frac{[\ (\text{cpm per 5 µl aliquot}) \times 4\ ]}{[\ \text{no. nmol added} + \text{carry-over from activated enzyme}\ ]}$$

The carry-over was 20 µl of a 10 mM solution ie. 200 nmol.

Each specific enzyme activity (nmol CO_2 fixed/ min/ mg protein) was then calculated from:

$$\text{Enzyme Specific Activity} = \frac{\text{EA}}{\text{BSA} \times \text{mg protein per assay vial}}$$

Finally, these rates were plotted (Lineweaver-Burk) against their respective bicarbonate concentrations (which were themselves corrected for the bicarbonate present in the activated enzyme solution), see fig. 3.13 for strain BC1. This yielded a K_m(bicarbonate)

value for the test enzyme. The $K_m(CO_2)$ was then calculated from this value by the following Henderson-Hasselbalch equation from Dawson (1974):

$$pH = pKa + \log_{10} \frac{[\text{ bicarbonate }]}{[\ CO_2\ ]}$$

Where the pKa for carbonic acid was 6.35.

2.10.2 K_m(ribulose-1,5-diphosphate) determination

The assay buffer, $MgCl_2$ and HCl were as for the $K_m(CO_2)$ determination (2.10.1).

Ribulose-1,5-diphosphate solutions of 12 mM, 6 mM, 3 mM, 1.5 mM and 0.75 mM were used, as well as 333 mM $NaH^{14}CO_3$ (4.8 µCi/µmol).

All reagents except ribulose-1,5-diphosphate were added as a pre-mix. Therefore each vial contained:

240 µl assay buffer
3 µl $MgCl_2$ (2 M)
27 µl $NaH^{14}CO_3$ (333 mM)
20 µl enzyme solution (2 mg/ml)

Incubation was for 15 min (activation period) before the reaction was initiated by the addition of 10 µl ribulose-1,5-diphosphate solution. The remainder of the protocol was as for the $K_m(CO_2)$ determinations (2.10.1).

Triplicate 5 µl samples of the 333 mM bicarbonate were added to 500 µl absorption solution for a bicarbonate specific activity determination.

A time course was established for each ribulose-1,5-bisphosphate concentration (see fig. 3.12 for strain BC1), as was previously done for each bicarbonate concentration (2.10.1). This yielded an enzyme activity (EA) in cpm/ min of assay for each ribulose-1,5-diphosphate concentration.

The bicarbonate specific activity (BSA) was calculated from:

$$\text{Bicarbonate Specific Activity (BSA)} = \frac{\frac{[\,(\text{cpm per 5 µl aliquote}) \times 27\,]}{5}}{9{,}000}$$

The specific enzyme activity (nmol CO_2/ min/ mg protein) for each substrate concentration was then calculated from:

$$\text{Specific Enzyme Activity} = \frac{\text{EA}}{\text{BSA} \times \text{mg protein per assay vial}}$$

Finally, these values were plotted (Lineweaver-Burk) to yield a K_m(ribulose-1,5-bisphosphate) for the test RuBisCO enzyme (see fig. 3.14 for strain BC1).

2.11 Assay of RuBisCO in polyacrylamide gels

The method employed was essentially that of Sani (1985).

RuBisCO assay buffer and ribulose-1,5-diphosphate solutions were as given earlier in description of enzyme assays (2.7).

Proteins were prepared in TEMMB buffer:

20 mM Tris-HCl (pH 8.0)

10 mM $MgCl_2.6H_2O$

50 mM $NaHCO_3$

1 mM EDTA

5 mM 2-mercaptoethanol

Cells in TEMMB were broken in the French Press and the soluble fraction obtained as described earlier (2.9). Up to 50 mg protein was layered onto a 0.2 M to 0.8 M sucrose step gradient in TEMMB. The gradients (2 ml of each 0.1 M sucrose solution) were prepared in 25 ml polycarbonate centrifuge tubes and, following soluble protein loading (upto 2 ml total volume), were centrifuged at 240,000 x g for 2.5 hours in a pre-cooled 8 x 25 titanium angle rotor at 4°C. The gradients were allowed to stand for 10 min before 1 ml fractions were collected from the bottom. These were monitored for protein by the Bradford (1976) method and assayed for RuBisCO activity as before (2.9). Active fractions were pooled and subjected to tube gel electrophoresis.

Following electrophoresis, the tube gels were frozen at -20°C and then sliced into 1 mm thick discs using a gel slicer (Micklelab Engineering Co., Gromshall, Surrey). Each disc was added to 200 µl RuBisCO assay mix in an eppendorf tube which was left shaking at 4°C for at least 3 hours to facilitate protein elution and full activation. The tubes were then incubated for 10 min at 30°C or 45°C (as appropriate), the reaction initiated with 20 µl (10 mM) ribulose-1,5-diphosphate solution and terminated 30 min later by the addition of 200 µl HCl (2 M). Unfixed bicarbonate was driven off and counts determined as described earlier.

2.12 Measurement of CO_2 uptake by whole cells

Cells used in these assays were prepared by two methods:

i) The term "growing cells" has been used to refer to cells that were taken from a culture in mid-exponential phase. Samples were added to a flask and directly assayed as described below.

ii) The term "resuspended cells" has been used to describe cells that had been harvested and resuspended in pH 1.6 medium to an optical density (440 nm) of approximately 0.15. This cell suspension was added to flasks and assayed as described below.

Stock solutions used were 36 mM $NaH^{14}CO_3$ (10.36 µCi/µmol) and absorption fluid.

15 ml of cell suspension were added to a 25 ml quick-fit conical flask containing a magnetic stirrer and a rubber bung used to seal the neck. This was then degassed for 10 min at 30°C or 45°C (as appropriate) with CO_2-free air.

The flask was clamped in a water bath above a heater-stirrer which maintained a constant temperature by means of a feed-back contact thermometer. The assay was initiated by the addition of an appropriate volume of 36 mM bicarbonate.

At 1 min time intervals, duplicate 1 ml samples were removed through a long needle using a 1 ml plastipak syringe (Becton-Dickinson, Dun Laoghaire, Co. Dublin). These samples were immediately filtered through 0.45 µm cellulose nitrate filters (Sartorius AG., W-3400, Goettingen, Germany) at a pressure of 10 bar and washed with 10 ml medium (pH 1.6). Finally, the filters were placed in 20 ml scintillation vials containing either 200 µl absorption fluid (total counts) or 500 µl hot ethanol + 200 µl 6 M phosphoric acid (fixed counts), as appropriate.

The whole process of sampling and filtering was completed in less than 10 seconds.

Triplicate 5 µl samples of 36 mM bicarbonate were retained for specific activity determination.

Protein determinations were made by two separate methods depending on the type of cell preparation used in the assay.

i) Resuspended cells:

During resuspension, triplicate 1 ml samples at $O.D._{440}$ were taken, centrifuged and protein determined by the Lowry *et al.* (1951) method.

ii) Growing cells:

Triplicate 50 ml samples of mid-log phase culture were filtered and protein determinations performed directly on the filters according to Lowry *et al.* (1951). Filters were also included with the protein standards (see 2.4).

For a regeneration period, where utilized, the assay flasks were incubated at 45°C in a shaking water bath and 50 µl additions of 1 M $FeSO_4$ were made every 30 min for 3 hours.

As a result of the acidic nature of the organisms studied, it was most appropriate to express the final assay concentrations of C_i used as a percentage (v/v) of CO_2 in air. The following calculations were therefore required:

1 mol of gas occupies 22.4 L at 25°C, 1 atmosphere.

Using Charles law: $\frac{V_1}{T_1} = \frac{V_2}{T_2}$ (V = volume) (T = temperature)

Therefore:

1 mol of gas occupies 22.8 L at 30°C, 1 atmosphere.

1 mol of gas occupies 23.9 L at 45°C, 1 atmosphere.

Since 1 µl of 36 mM bicarbonate contains 36 nmol:

At 30°C, 1 µl 36 mM bicarbonate occupies 8.208×10^{-4} ml

At 45°C, 1 µl 36 mM bicarbonate occupies 8.604×10^{-4} ml

Bicarbonate additions made (µl) were 10, 15, 25, 50, 250, 500 and the vial assay volume was 37 ml.

These additions correspond to final CO_2 percentages (v/v) of:

At 30°C; 0.022, 0.033, 0.055, 0.111, 0.555, 1.109 (respectively)

At 45°C; 0.023, 0.035, 0.058, 0.116, 0.581, 1.163 (respectively)

Bicarbonate specific activity (BSA) was calculated from:

$$\text{BSA (cpm/ nmol } CO_2\text{)} = \frac{(\text{ cpm per 5 µl aliquot })}{(\text{ 5 x 36 })}$$

Background was then subtracted.

The whole cell uptake rate (cpm/ min of assay) was calculated by plotting cpm (minus background) against time.

Finally, the whole cell specific uptake rate was calculated from the equation below:

$$\text{SPECIFIC UPTAKE RATE (nmol } CO_2\text{/ min/ mg protein)} = \frac{[\text{ whole cell uptake rate (cpm/ min) }]}{[\text{ BSA x (protein per 1 ml sample) }]}$$

2.13 Membrane preparation

All stages were performed at 4°C.

T. ferrooxidans cells were harvested, separated into two batches and lysed in either 10 mM MOPS buffer (pH 6.5) or in suphuric acid (pH 2.5).

Immediately after French pressing, the pH 2.5 lysate was re-adjusted to pH 2.5 with 5% (v/v) sulphuric acid.

Two centrifugations were performed at 10,000 x g to remove cell debris and unbroken cells.

The lysate was centrifuged at 104,000 x g for 2 hours using a Beckman Sw28 rotor to yield the soluble fraction (supernatant) and membrane fraction (pellet). The membrane fraction was further washed by repeating the above spin in appropriate buffer.

Samples were further analysed by SDS-PAGE and by spectrophotometry.

2.14 Spectrophotometry

A Beckman DU-70 spectrophotometer was used to obtain *T. ferrooxidans* cytochrome absorption spectra.

2.15 Protein determinations

Measurement of protein from whole cells was performed using the method of Lowry *et al.* (1951).

Protein in soluble extracts was performed using the method of Bradford (1976).

RESULTS AND DISCUSSION

CHAPTER 3

The effect of CO_2 on bacterial growth and enzyme activities.

3.1 Introduction

A number of Gram positive, moderately thermophilic bacteria have been described that are capable of both iron- and sulphur-oxidation (Norris, 1990; Karavaiko, 1988; Brierley, 1978). Although some of these organisms are sufficiently different to justify classification in different genera (Lane *et al.*, 1992) all have an inherent requirement for CO_2 enriched air in order to grow well and oxidize iron rapidly during autotrophic growth (Marsh and Norris, 1983a; Norris, 1990).

Strain BC1, which closely resembles *Sulfobacillus thermosulfidooxidans*, is one of the most studied of these bacteria and so was chosen as the representative moderate thermophile for the purpose of these studies (see section 1.2.2).

The control organism for this study, *T. ferrooxidans* (see section 1.2.1), has demonstrated similar iron-oxidation (Kelly and Jones, 1978) and mineral solubilization (Norris, 1989b) rates with both air levels and enhanced levels of CO_2. Furthermore, this organism has been used extensively in bacterial oxidation process development and as such is a standard with which potentially useful bacteria can be compared (Norris, 1989b).

3.2 The effect of CO_2 on the growth of strain BC1 and *T. ferrooxidans*.

Both strain BC1 (fig. 3.1) and *T. ferrooxidans* (fig. 3.2) were grown in 1 litre reactors at their optimal temperatures of 45°C and 30°C respectively.

Ferrous iron was used as a defined, soluble substrate in preference to mineral sulphides. The complexities of using a solid substrate of variable composition with a mixture of iron and sulphur as the energy sources would have precluded simple, reproducible experimentation. In the case of strain BC1, 0.5 mM tetrathionate was added to overcome

the organism's inability to use sulphate as a source of sulphur for biosynthesis (Norris and Barr, 1985; Wood and Kelly, 1983).

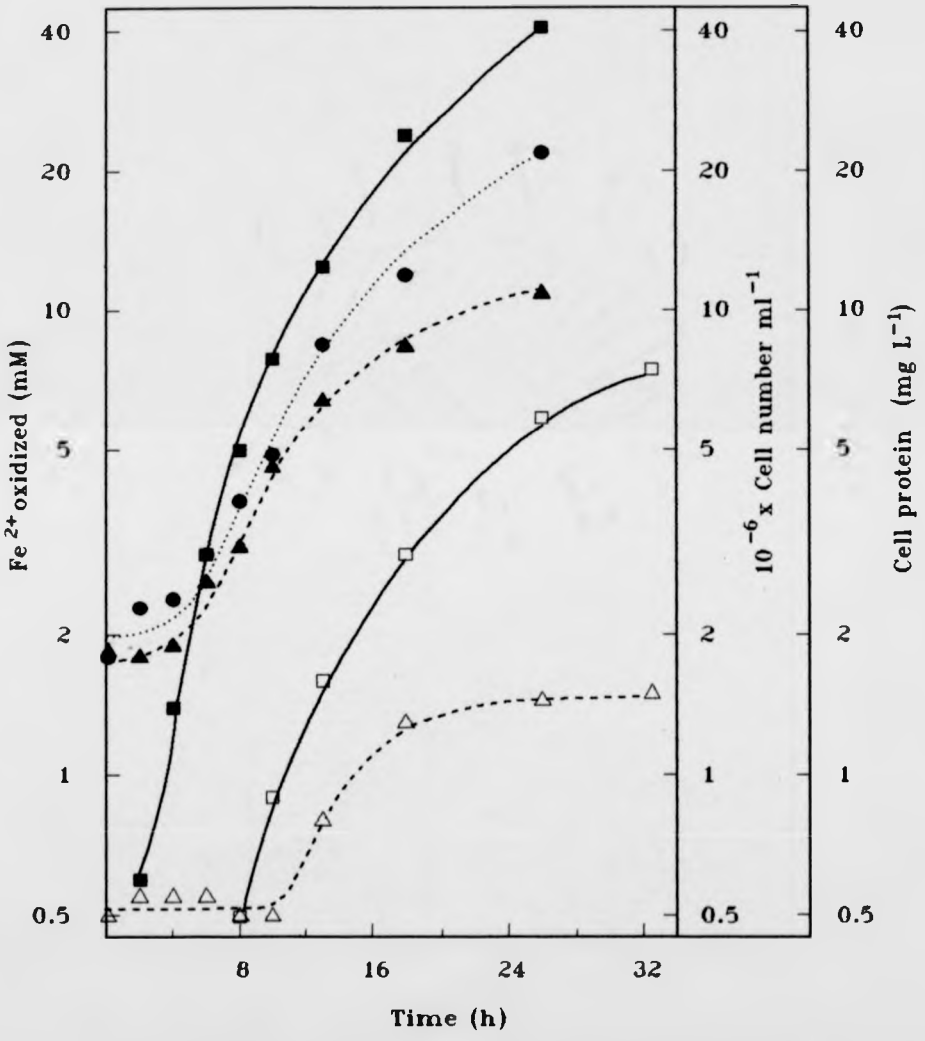
Growth of strain BC1 (fig. 3.1) supplied with 1% (v/v) CO_2 occurred with a doubling time (measured as the logarithmic rate of iron-oxidation) of 3 hours. Both cell protein and numbers closely paralleled this growth rate in the short exponential phase. This doubling time was shorter than other reported values of 6 to 7 hours (Norris and Barr, 1985; Marsh and Norris, 1983a) and 10.5 hours (Wood and Kelly, 1983) for this organism during autotrophic growth with similar levels of CO_2, and was more comparable with previously quoted chemolithoheterotrophic (Norris and Barr, 1985; Marsh and Norris, 1983a) and mixotrophic (Wood and Kelly, 1983) growth rates. The most likely explanation for this was a result of more efficient mass transfer in the 1 litre reactors employed for this work than in the shake flasks used in the above reports. Furthermore, some of the growth rates described earlier were taken from a later stage in the growth curve, i.e. after the most rapid initial burst of growth had passed.

After 14 hours incubation, the notable decrease in growth rate (seen in cell protein and numbers) possibly resulted from an increasing ferric: ferrous iron ratio.

In contrast, and following a considerably longer lag phase, air levels of CO_2 supported an initial, though abrupt, doubling time of 4 hours for strain BC1. However, cell numbers indicated that this initial iron-oxidation rate was only associated with a single population doubling, thereafter, iron-oxidation was performed by a constant number of cells. It was uncertain as to why these conditions only supported a single population doubling since strain BC1 has been maintained autotrophically with air levels of CO_2 for over 50 serial sub-cultures. One explanation may be that under such conditions the air-gassed

Figure 3.1

Iron oxidation and growth by strain BC1 grown in 1 litre reactors at 45°C with 50 mM ferrous iron and 0.5 mM tetrathionate. Carbon dioxide was supplied at either air levels, or 1% (v/v) in air as indicated.



closed symbols represent CO_2 supplied at 1% (v/v) in air
open symbols represent CO_2 supplied at air levels

■ □ Fe^{2+} oxidized (mM)

▲ △ 10^{-6} x Cell number ml^{-1}

● Cell protein (mg L^{-1})

cells were growing mixotrophically, utilizing carbon from both CO_2 and a possible contaminating source (e.g. from media salts) which was rapidly exhausted. Evidence for this type of oligocarbophilic growth has been observed with *Thiobacillus acidophilus* which demonstrated the ability to scavenge both carbon and energy from unamended 9K basal salts medium (Arkesteyn, 1980). Alternatively, it may have been a reflection of the metabolic state of the cells which possibly were barely capable of withstanding the combined pressures of an increasing ferric iron concentration and a possible extra "effort" required to assimilate CO_2 when its concentration was not enhanced.

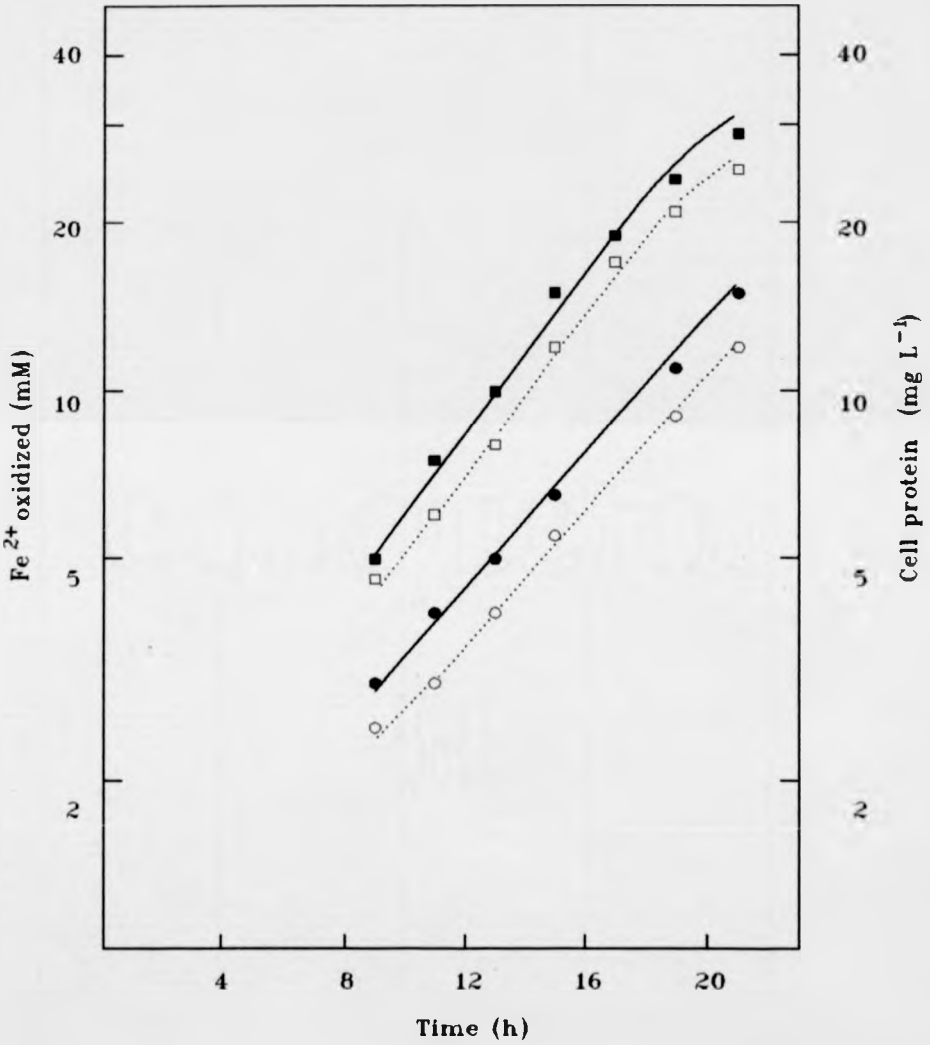
No cell protein values were plotted for growth under air owing to the extremely poor biomass yields obtained and consequent technical problems in obtaining accurate data.

Figure 3.2 illustrates the effect that the CO_2 concentration had on both iron-oxidation rate and cell protein yield of the control organism *T. ferrooxidans*. Gassing with either air or 1% (v/v) CO_2 in air allowed identical doubling times of 4 hours, although a slightly longer lag phase was observed for the air grown cells.

Cell numbers were not counted for *T. ferrooxidans* because the small size of this organism relative to strain BC1 made the counting extremely difficult and time consuming, while the iron-oxidation and protein data provided a convincing record of the lack of influence a reduction in the CO_2 concentration had on growth. This was supportive of earlier work by Norris (1989b) and Kelly and Jones (1978), though contradictory to one report by Holuigue *et al.* (1987) in which notable growth rate differences were observed over a similar range of CO_2 concentrations. However, it should be noted that this latter report utilized a strain of *T. ferrooxidans* that exhibited some further unique features (see section 3.6).

Figure 3.2

Iron oxidation and growth of *T. ferrooxidans* grown in 1 litre reactors at 30°C with 50 mM ferrous iron. Carbon dioxide was supplied at either air levels, or 1% (v/v) in air as indicated.



closed symbols represent CO_2 supplied at 1% (v/v) in air

open symbols represent CO_2 supplied at air levels

■ □ Fe^{2+} oxidized (mM)

● ○ Cell protein (mg L^{-1})

Similar growth yields of approximately 300 mg protein/ mole ferrous iron oxidized were obtained for both strain BC1 and *T. ferrooxidans* under conditions where growth was not CO_2-limited. These values were slightly higher than those quoted by Wood and Kelly (1983) for several moderate thermophiles and by Kelly and Jones (1978) for *T. ferrooxidans*.

3.3 Cell protein profiles of strain BC1 and *T. ferrooxidans* grown under different CO_2 conditions.

Following growth in 1 litre reactors (fig. 3.1 and 3.2), cells were harvested during mid-exponential phase, lysed and soluble proteins subjected to 10% (w/v) SDS-PAGE (see fig. 3.3). Duplicate gels were run: one Coomassie stained for protein and the other western blotted using anti-*Rhodobacter sphaeroides* (form I) RuBisCO antibodies.

In all four Coomassie stained tracks a band corresponding to the RuBisCO large sub-unit, confirmed by the western blot (fig. 3.3), was evident. For strain BC1 and *T. ferrooxidans* these polypeptides had apparent molecular weights of 56 kDa and 54 kDa respectively and were of typical size for bacterial RuBisCO large sub-units (see Tabita, 1988).

Densitometer analysis of the large sub-unit polypeptide bands on these gels (table 3.1), followed by correction for loading differences, indicated that strain BC1 had increased RuBisCO production 3.4-fold during growth under air. By comparison, *T. ferrooxidans* demonstrated a 2.5-fold derepression. Should both organisms be capable of similar maximal levels of RuBisCO derepression, this may have suggested that CO_2 was more limiting in air-grown cells of strain BC1 than in air-grown *T. ferrobxidans*. A similar range of derepression has been observed in the obligate chemolithoautotroph, *Thiobacillus neapolitanus* (Beudeker *et al.*, 1980) and in other autotrophic bacteria (see

Figure 3.3 10% (w/v) SDS-PAGE, followed by an anti-RuBisCO western blot of soluble protein extract from strain BC1 and *T. ferrooxidans*.

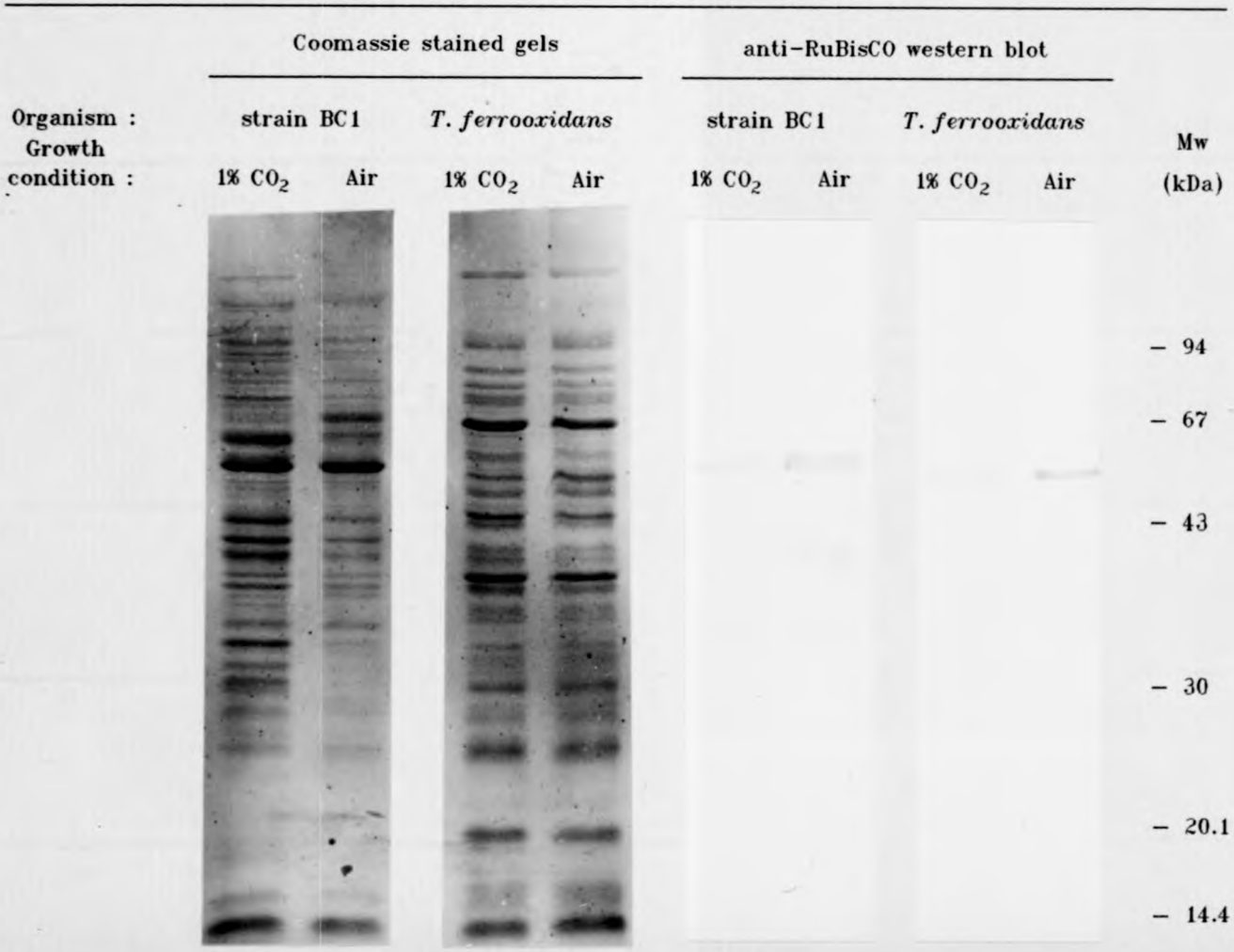


Table 3.1 Densitometer analysis of figure 3.3 (Coomassie stained gels).

Organism	Growth condition	RuBisCO (lsu) peak height	Protein correction *	Derepression ratio
T. ferrooxidans	1% CO_2	1.89	1.89	1
	AIR	3.92	4.74	2.5
Strain BC1	1% CO_2	5.53	5.53	1
	AIR	4.77	18.8	3.4

* The loading differences between tracks were standardized using the densitometer-integrated peaks of those protein bands unaffected by the CO_2 concentration.

Tabita, 1988).

In contrast to the RuBisCO data, there was no obvious increase of a 32 to 36 kDa polypeptide, that may have corresponded to the sub-units of PRK (see section 1.3.3), during autotrophic growth with air in either organism.

Similarly, no evidence was provided for an earlier observation with strain BC1 (Norris, 1989b) in which a 52 kDa protein appeared to be present in an inversely proportional concentration to that of the RuBisCO large sub-unit with differing CO_2 concentrations. It had been suggested that this 52 kDa protein may have represented a post-translational modification system of RuBisCO in strain BC1, analogous to that in *Chromatium* (see Tabita, 1988).

3.4 The effect of CO_2 concentration on enzyme activities.

Initial experiments used permeabilized cells, removing the necessity for cell lysis. A literature search suggested the use of two such permeabilizing agents: the non-ionic surfactant Triton X-100 (Smith *et al.*, 1980; Schnaitman, 1971; Gonzalez-Manas *et al.*, 1990), and the ionic detergent cetyltrimethylammonium bromide (Leadbeater *et al.*, 1982), abbreviated to CTAB.

Both detergents were tested over a range of concentrations (fig. 3.4 and fig. 3.5) in order to ascertain their suitability and optimal working concentration for each organism.

Cells were grown autotrophically with 1% (v/v) CO_2 in air and the RuBisCO activities were determined.

In the case of CTAB (fig. 3.4) a sharp peak of RuBisCO activity was observed over a narrow range of test concentrations (0.03 to 0.05% w/v), for both strain BC1 and *T. ferrooxidans*. By contrast, optimal RuBisCO activity was achieved over a much wider spectrum with Triton X-100 (fig. 3.5), making this detergent a more suitable choice than CTAB as the permeabilization agent.

Figure 3.4

Optimization of the cetyltrimethylammonium bromide (CTAB) concentration for use in the permeabilized cell enzyme assays. Both organisms were grown autotrophically with 1% (v/v) CO_2 in air.

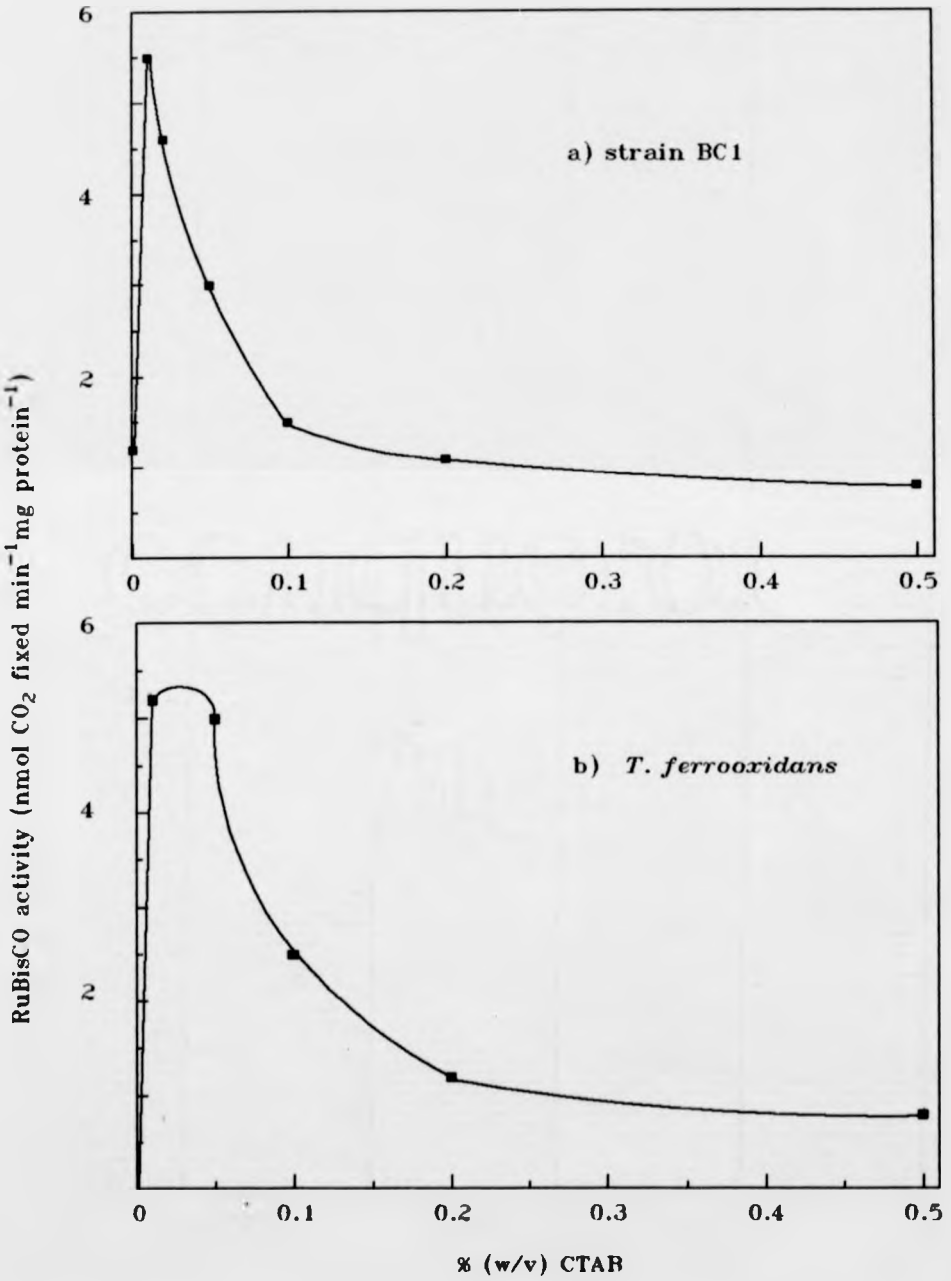
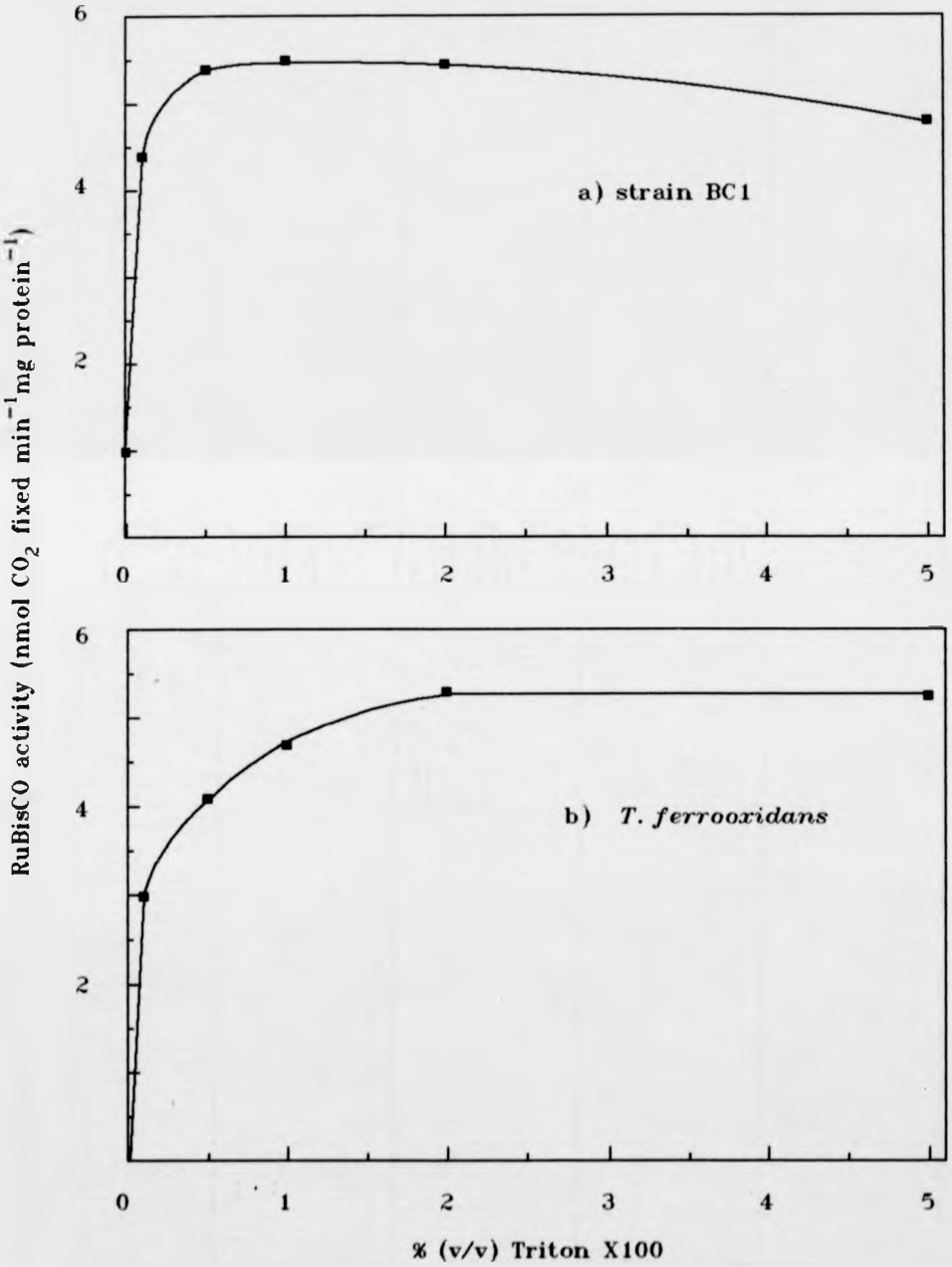


Figure 3.5

Optimization of the Triton X 100 concentration for use in the permeabilized cell enzyme assays. Both organisms were grown autotrophically with 1% (v/v) CO_2 in air.



Consequently Triton X-100 was selected, and the optimal assay concentrations of 1% (v/v) and 3% (v/v) were chosen for strain BC1 and *T. ferrooxidans* respectively. In the case of strain BC1, water as opposed to detergent was sufficient to allow approximately 20% total RuBisCO activity (fig. 3.4 and fig. 3.5). In contrast, no activity was observed in the absence of detergent with *T. ferrooxidans*. These differences probably reflected lysis of some strain BC1 cells, a tendency apparent from earlier growth studies (Wood and Kelly, 1983).

Enzyme activities were also determined using cell-free extracts.

Table 3.2 summarizes the effect of the CO_2 concentration during growth on the activities of the major enzymes involved in CO_2 fixation for permeabilized cells of both strain BC1 and *T. ferrooxidans*. Each organism demonstrated a two- to three-fold increase in RuBisCO activity under air. This was in accordance with the densitometer analysis of protein profiles (table 3.1) and similar to reported values for other bacteria (*Thiobacillus versutus*: Smith *et al.*, 1980; *Thiobacillus neapolitanus*: Beudeker *et al.*, 1980).

The maximum rates of CO_2 fixation by RuBisCO were similar for both strain BC1 and *T. ferrooxidans* and were comparable with those obtained by Wood and Kelly (1985) for several moderate thermophiles.

Phosphoribulokinase activities were similar in the two organisms, although in contrast to the RuBisCO activities, they did not appear to be affected by the CO_2 concentration. This suggested that in both organisms RuBisCO and PRK were independently regulated. In *Alcaligenes eutrophus* the two enzymes are known to form part of an operon (Leadbeater *et al.*, 1982; Bowien *et al.*, 1990) and as such are co-ordinately regulated.

A ten-fold difference in phosphoenolpyruvate (PEP) carboxylase activity was apparent between permeabilized cells of strain BC1 and *T. ferrooxidans*. Although no appreciable differences in activity were

Table 3.2 Enzyme activities in permeabilized cells. Cell pellets were resuspended in the appropriate Triton X 100 concentration (see fig. 3.5) for each organism and the permeabilized cell enzyme activities determined.

	T. ferrooxidans		Strain BC1	
Growth condition :	1% CO_2	Air	1% CO_2	Air
RuBisCO	5.6 (±2.5)	12.5 (±5.6)	5.3 (±2.4)	13.0 (±5.8)
Phosphoribulokinase	25.0 (±11.2)	23.0 (±10.4)	24.0 (±10.8)	22.0 (±10.0)
PEP carboxylase	8.0 (±3.6)	6.0 (±2.7)	0.8 (±0.4)	0.6 (±0.3)
Pyruvate carboxylase	N.D.	N.D.	N.D.	N.D.

N.D. not detectable

Enzyme activities (nmol CO_2 fixed min^{-1}mg $protein^{-1}$) were the mean of five independent experiments.

Standard deviation is given in brackets

Table 3.3 Enzyme activities in cell-free extracts.

	T. ferrooxidans		Strain BC1	
Growth condition :	1% CO_2	Air	1% CO_2	Air
RuBisCO	4.8 (±1.9)	12.4 (±5.1)	5.2 (±2.1)	14.1 (±5.7)
PEP carboxylase	6.4 (±2.6)	3.6 (±1.5)	4.1 (±1.7)	4.0 (±1.6)

Enzyme activities (nmol CO_2 fixed min^{-1} mg $protein^{-1}$) were the mean of three independent experiments.

Standard deviation is given in brackets.

observed with respect to the CO_2 concentration, this was initially a major difference between the two organisms. However, a comparison of cell-free enzyme activities (table 3.3) demonstrated that there was very little inter-organism difference in PEP carboxylase activity. The observed differences for PEP carboxylase activity in permeabilized cell and cell-free extracts of strain BC1 may have been accounted for by the presence of an auto-inhibitor which was removed or inactivated during extract preparation. Alternatively, the PEP carboxylase enzyme in strain BC1 may have been exceptionally sensitive to the Triton X-100 detergent.

No activity for pyruvate carboxylase was detected in permeabilized cells for either organism.

With the exception of PEP carboxylase activity in strain BC1, similar maximal enzyme activities were observed for both Triton X-100 permeabilized cells and cell-free extracts.

The above enzyme data suggested that there were no significant differences in maximal enzyme activities between strain BC1 and the control organism *T. ferrooxidans*; at least not one that could account for the ability of one organism, but not the other, to grow autotrophically with air levels of CO_2. Although no significant differences were observed for the maximal enzyme rates (table 3.2), the possibility that, under physiological (as opposed to assay) conditions, CO_2 fixation was limited through the poor affinity of an enzyme in the thermophile also had to be examined.

The Calvin cycle had been assumed to be the major CO_2 fixation pathway in the moderate thermophiles through demonstration of combined RuBisCO and PRK activity (table 3.2), in conjunction with earlier work by Wood and Kelly (1983 and 1985). The latter had

calculated that CO_2 fixation rates by RuBisCO were sufficient to support autotrophic growth of these organisms.

Consequently, should a poor affinity CO_2 fixation enzyme exist in strain BC1, the most likely candidate would be RuBisCO. This was for a number of reasons.

RuBisCO is the first enzyme in the Calvin cycle and as such is responsible for regulation of the carbon flux entering a cell (see fig. 1.1). Furthermore, that RuBisCO exists with varying affinities, especially for CO_2, has been well documented (see table 1.2).

Despite having comparable maximal enzyme activities to that of *T. ferrooxidans* (tables 3.2 and 3.3), it was therefore possible that a low affinity RuBisCO in strain BC1 may have been responsible for the inferior autotrophic growth capability of this organism with air levels of CO_2.

Accordingly, the substrate affinities of this enzyme from strain BC1 were compared with that from *T. ferrooxidans*.

3.5 Purification of RuBisCO from strain BC1 and *T. ferrooxidans*.

The purification procedure for RuBisCO from both organisms was by an identical protocol. Consequently, only the separate stages for strain BC1 are described.

Strain BC1 biomass was grown autotrophically by batch culture with 1% (v/v) CO_2 (as opposed to air levels) in 20 litre vessels. Although RuBisCO, as a percentage of the total cell protein was lower under such conditions (fig. 3.3), this was necessary because of the extremely poor biomass yields observed for this organism grown autotrophically with air levels of CO_2 (fig. 3.1).

The first stage involved the use of ammonium sulphate precipitation. This method has been frequently applied as the primary stage in

RuBisCO purifications (Lanaras *et al.*, 1991; Holuigue *et al.*, 1987; Smrcka *et al.*, 1991) and occasionally as a supplementary stage to protamine sulphate precipitation (Mikulik *et al.*, 1992). In some cases polyethylene glycol treatment and magnesium chloride precipitation have been used as an alternative (Heda and Madigan, 1989), but all variations appeared to allow similar recovery yields and purification factors.

Thirty to fifty five percent (w/v) ammonium sulphate precipitation of strain BC1 soluble extract (fig. 3.6) allowed a 1.4-fold purification (table 3.4) of the RuBisCO protein.

Following dialysis and ultrafiltration, 4 ml of protein extract were loaded onto each of two sucrose step gradients (0.2 M to 0.8 M) and centrifuged (fig. 3.7). Following activity testing, fraction 35 was then pooled from both gradients and dialysed against isolation buffer as before. This allowed separation of RuBisCO from the bulk of the remaining protein and resulted in a further purification of over ten-fold (table 3.4). Similar orders of purification have been observed following sucrose density centrifugation for *Thiobacillus thyasiris* (Lanaras *et al.*, 1991) and *Chromatium tepidum* (Heda and Madigan, 1989).

Both step sucrose and linear type gradients were assessed for use in the RuBisCO purification. However, the former was chosen in preference because it provided a greater concentration of the RuBisCO protein over fewer fractions.

Four to thirty percent (w/v) native gradient gel electrophoresis of sucrose gradient fractions 30 to 40 (fig. 3.8a) clearly indicated the accumulation of a protein (approximately 550 kDa) whose concentration correlated with that of RuBisCO activity.

The three major protein bands in fig. 3.8a (designated 1, 2 and 3) were excised from the native gel and subjected to 10% (w/v) SDS-PAGE. Following silver staining, these proteins were revealed to

Figure 3.6 Ammonium sulphate precipitation of strain BC1 RuBisCO. Following precipitation into 0–30, 30–55, 55–70 and 70–100% (w/v) ammonium sulphate fractions, each was dialysed against isolation buffer and assayed for protein and RuBisCO activity.

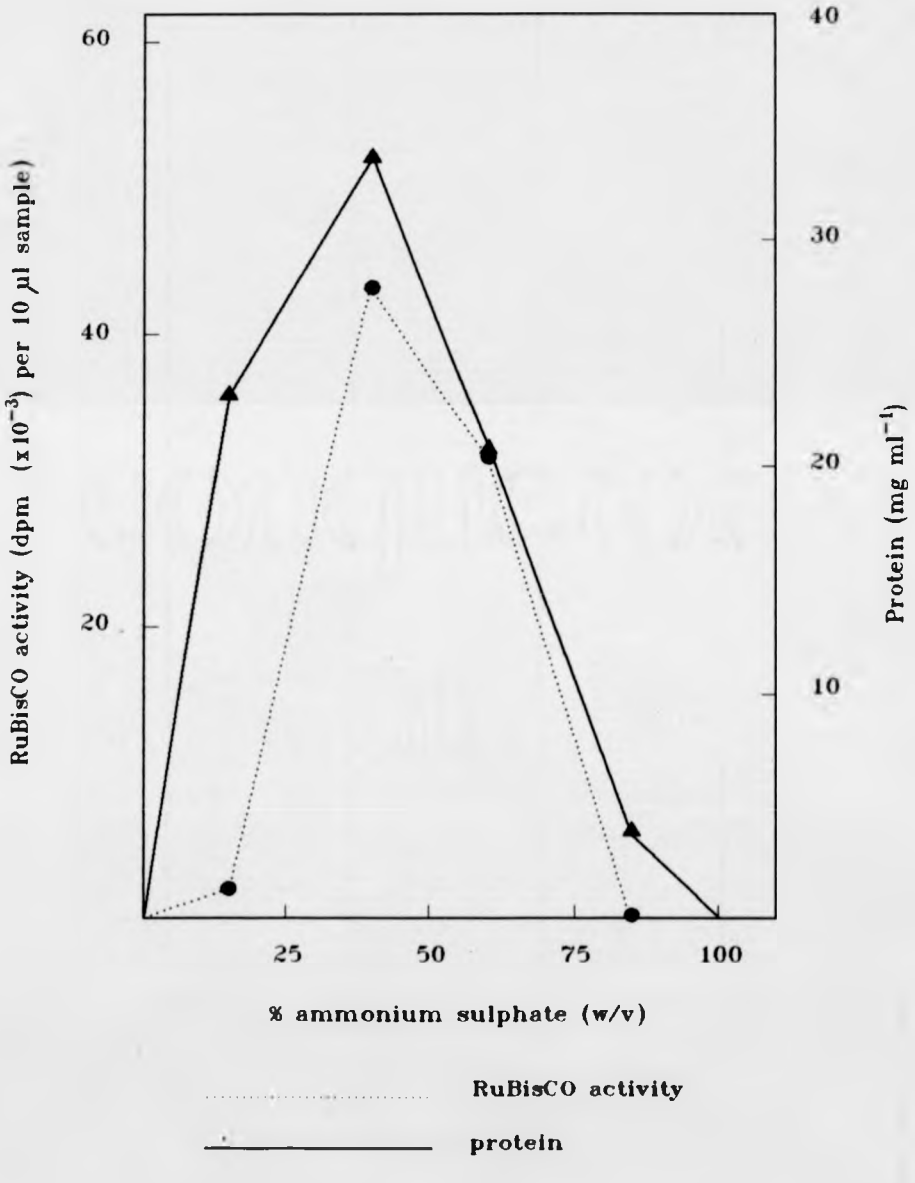


Figure 3.7 Sucrose gradient centrifugation of strain BC1 RuBisCO. The 30-55% (w/v) ammonium sulphate fraction was centrifuged in 0.2 to 0.8 M sucrose step gradients. 1 ml fractions were collected from the bottom and assayed for protein and RuBisCO activity. The most active fractions were pooled and dialysed.

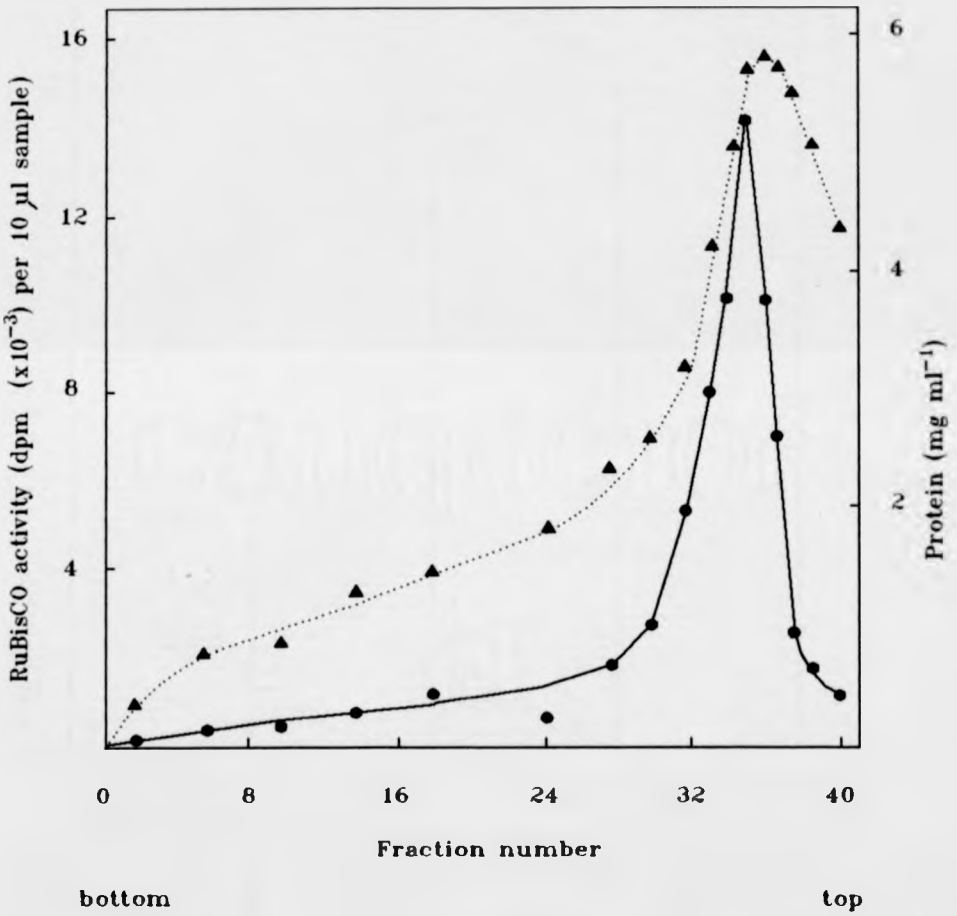
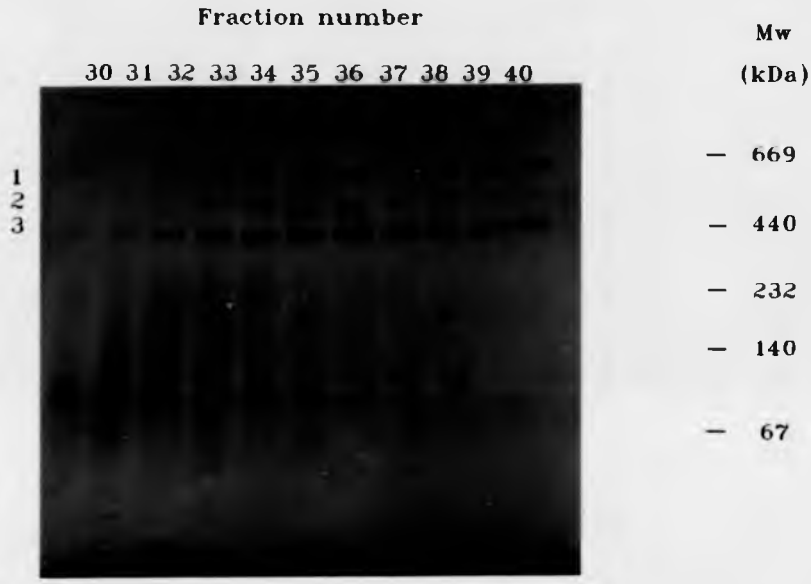
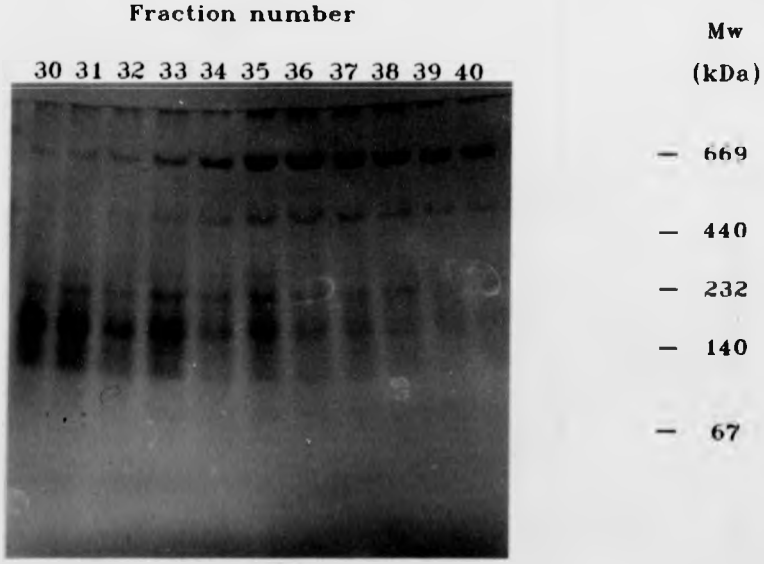


Figure 3.8 Native gradient gel electrophoresis of sucrose gradient fractions. Soluble protein extract from sucrose gradient fractions 30 to 40 (see fig. 3.7 for strain BC1) was subjected to 4–30% (w/v) native gel electrophoresis.

a) Strain BC1



b) *T. ferrooxidans*



comprise sub-units of 35kDa, 56kDa and 50kDa respectively (results not illustrated). Although protein 2 was therefore the most likely to be the RuBisCO from strain BC1 (see also fig. 3.10, RuBisCO standard included), protein 3 may have represented the RuBisCO protein devoid of small sub-units. Dissociation of small sub-units from the native enzyme during purification has been well documented (Holuigue *et al.*, 1989; Taylor and Dow, 1980), and in several cases has resulted in the incorrect conclusion that multiple forms of RuBisCO have existed within a given bacterium (see Tabita, 1988).

The final purification stage involved FPLC with a Green-A dye ligand column (fig. 3.9). Fractions 21 and 22 contained the highest RuBisCO activity and were subsequently pooled and dialysed as before, although this time against post-sucrose gradient buffer. The FPLC stage most notably removed a protein of approximately 600 kDa (comparison of tracks 5 and 6, fig. 3.10) and had allowed an over-all purification factor of 64 for the strain BC1 RuBisCO. Ultrafiltration finally produced an enzyme solution of approximately 2 mg/ml which was subsequently used for the K_m determinations. A summary of the purification protocol for strain BC1 and *T. ferrooxidans* is presented in table 3.4.1 and 3.4.2, respectively.

Unfortunately, the RuBisCO purification from *T. ferrooxidans* was not as successful as that from strain BC1. Comparison of the specifc activity column (table 3.4.2) and track numbers 3 and 4 (fig. 10) indicated that the protein was denatured at a stage either during or following FPLC. As a result of this it was necessary to use the pooled, active sucrose gradient fracions (table 3.4.2) for the RuBisCO K_m determinations of this organism.

Denaturation of the *T. ferrooxidans* RuBisCO may have resulted from a reduced thermotolerance of the enzyme during the FPLC stage, during which only limited cooling (10°C) was achieved. Alternatively, the protein may have been less stable in the relatively high salt

Figure 3.9 FPLC purification of strain BC1 RuBisCO with a Green-A column. A linear salt gradient (0 to 2 M KCl) was used to elute the bound proteins in 3 ml fractions which were assayed for RuBisCO activity and protein. The most active fractions were dialysed against post-sucrose gradient buffer and ultrafiltrated prior to K_m determinations.

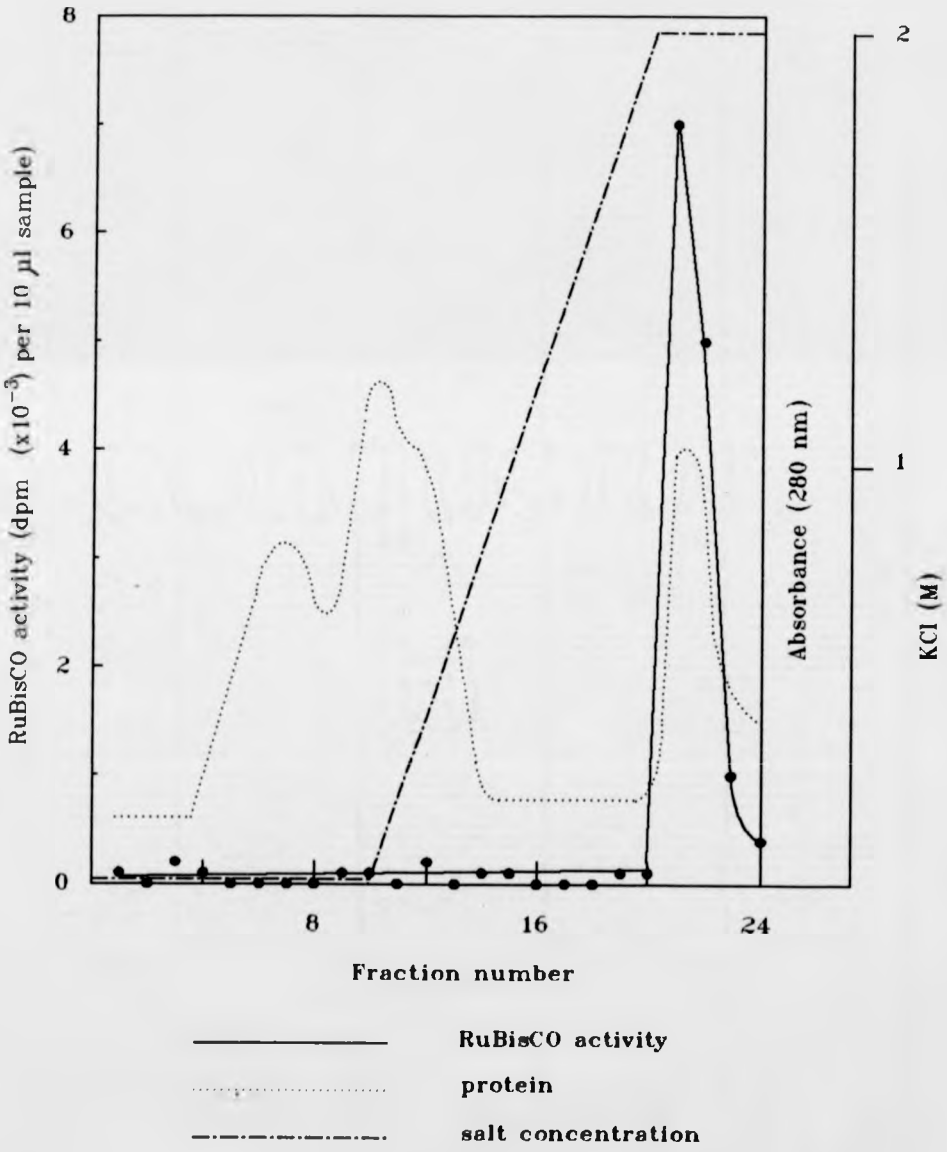
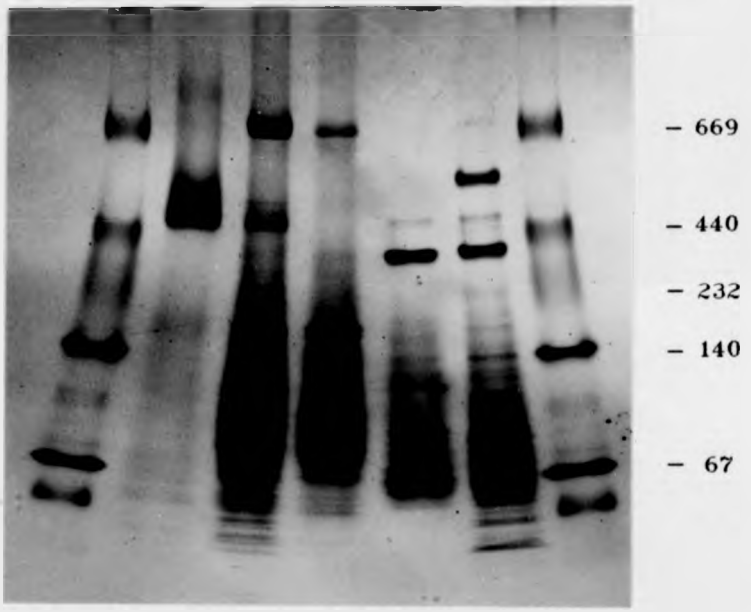


Figure 3.10

3 to 40% (w/v) native gel electrophoresis of pooled post-sucrose gradient and post-FPLC RuBisCO fractions from strain BC1 and *T. ferrooxidans*.



Track number

1 Mw markers
2 Spinach RuBisCO (Sigma)
3 *T. ferrooxidans*. Pooled, active, post-sucrose gradient fractions
4 *T. ferrooxidans*. Pooled, active, post-FPLC fractions
5 Strain BC1. Pooled, active, post-FPLC fractions
6 Strain BC1. Pooled, active, post-sucrose gradient fractions
7 Mw markers

Table 3.4.1 Summary of the purification data for RuBisCO from strain BC1.

Purification step	Total protein (mg)	Total activity, *a*	Specific activity, *b*	Yield	Purification (-fold)
cell-free extract	450	2260	5	100	1.0
membrane-free extract	420	2100	5	93	1.0
30-55 % ammonium sulphate	206	1445	7	64	1.4
pooled, active sucrose fractions	10	810	81	36	16.2
pooled, active Green-A fractions	2.1	634	302*	28	64

a nmol CO_2 fixed/ min

b nmol CO_2 fixed/ min/ mg protein

* Denotes fraction used for K_m estimations

Table 3.4.2 Summary of the purification data for RuBisCO from *T. ferrooxidans*.

Purification step	Total protein (mg)	Total activity, *a*	Specific activity, *b*	Yield	Purification (-fold)
cell-free extract	240	2880	12	100	1.0
membrane-free extract	218	2180	10	76	0.8
30-55 % ammonium sulphate	126	1890	15	66	1.3
pooled, active sucrose fractions	23	1449	63 *	50	5.3
pooled, active Green-A fractions	3	15	5	0.5	0.4

a nmol CO_2 fixed/ min

b nmol CO_2 fixed/ min/ mg protein

* Denotes fraction used for K_m estimations

concentration required for FPLC elution than its counterpart from strain BC1.

3.6 RuBisCO affinity determinations.

Following partial RuBisCO purification from both bacterial sources, the substrate affinities of these enzymes were investigated.

Enzyme was incubated with a range of either bicarbonate (0.4 to 5.6 mM) or ribulose-1,5-bisphosphate (0.025 to 0.4 mM) concentrations and a time course established with each concentration (illustrated for strain BC1 RuBisCO; see fig. 3.11 for bicarbonate and 3.12 for ribulose-1,5-bisphosphate).

Since the RuBisCO enzyme had to be pre-activated with bicarbonate and magnesium, each reaction was initiated by the addition of ribulose-1,5-bisphosphate (post sucrose gradient buffer in the case of the controls).

The maximum rates for each concentration were then calculated and the control rate subtracted. These rates were then plotted, Lineweaver-Burk style, against their corresponding substrate concentrations and the K_m(bicarbonate) and K_m(ribulose-1,5-bisphosphate) determined for each organism:

Fig. 3.13 and fig. 3.14 for strain BC1.

Fig. 3.15 and fig. 3.16 for *T. ferrooxidans*.

Fig. 3.17 and fig. 3.18 for the control, pea.

Pea (membrane-free, stromal extract) was included as a RuBisCO with well documented substrate affinities.

In the case of the bicarbonate time courses, these were firstly corrected for the bicarbonate carry-over present in the enzyme solution (post-sucrose gradient buffer) and then plotted against their

corrected concentrations. The $K_m(CO_2)$ was then calculated from K_m(bicarbonate) according to Dawson (1974).

The RuBisCO substrate affinity work has been summarized in table 3.5.

The respective mean $K_m(CO_2)$ values of 43.5 µm and 47 µm for strain BC1 and *T. ferrooxidans* were very similar, as were their respective mean K_m(ribulose-1,5-bisphosphate) values of 67.5 µm and 85.5 µm.

These data therefore clearly indicated that there was very little difference between the substrate affinities of these two bacterial RuBisCOs with that from strain BC1 exhibiting the slightly higher substrate affinities for both CO_2 and ribulose-1,5-bisphosphate.

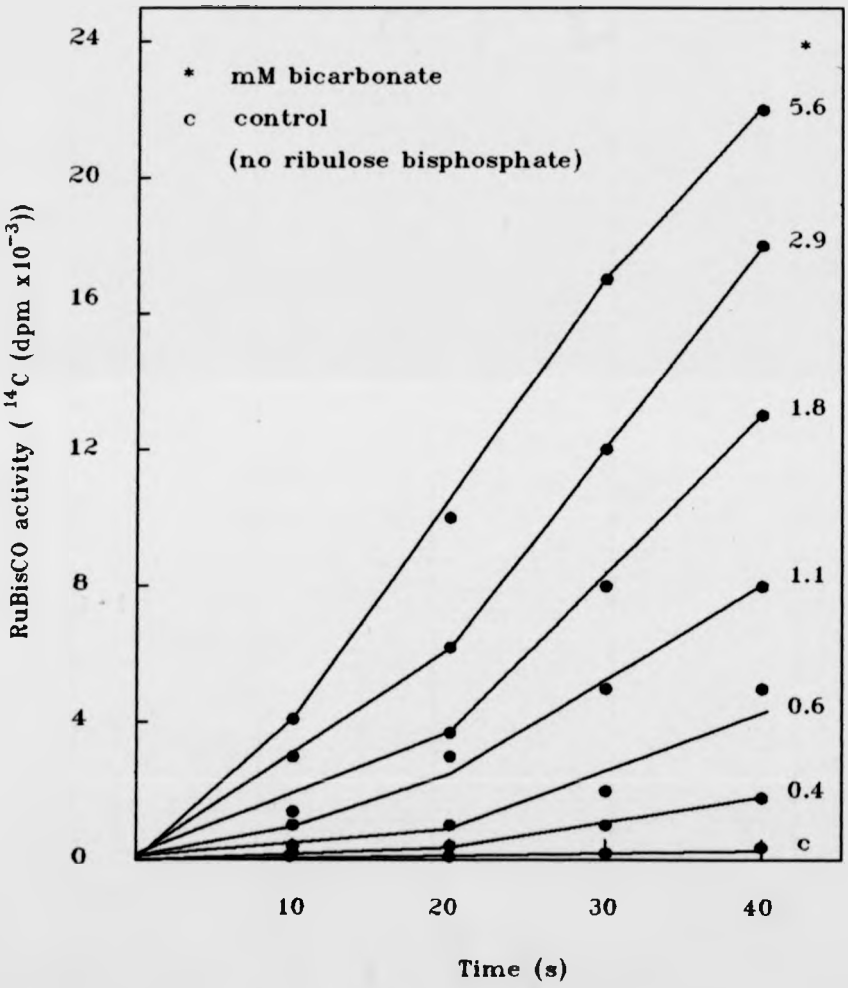
The $K_m(CO_2)$ values for the two enzymes appeared to be most closely related to those of form I type RuBisCOs from other sources (see table 1.2), but with the notable exception of the cyanobacteria (see also section 1.3.3).

Although the K_m(ribulose-1,5-bisphosphate) for *T. ferrooxidans* was very similar to that quoted by Holuigue *et al.* (1987), table 1.2, the $K_m(CO_2)$ obtained was higher. However, it should be noted that the strain used by Holuigue *et al.* (1987) also exhibited a lower RuBisCO specific activity during autotrophic growth with air levels of CO_2 than with 0.35% and 5.40% (v/v) CO_2.

Finally, the control RuBisCO from pea gave respective K_m values for CO_2 and ribulose-1,5-bisphosphate of 23.5 µm and 30 µm. These values are very close to those quoted by Yeoh *et al.* (1981) for C_3 terrestrial plants (see table 1.2).

Figure 3.11 Strain BC1 RuBisCO $K_m(CO_2)$ determination (time course experiments).

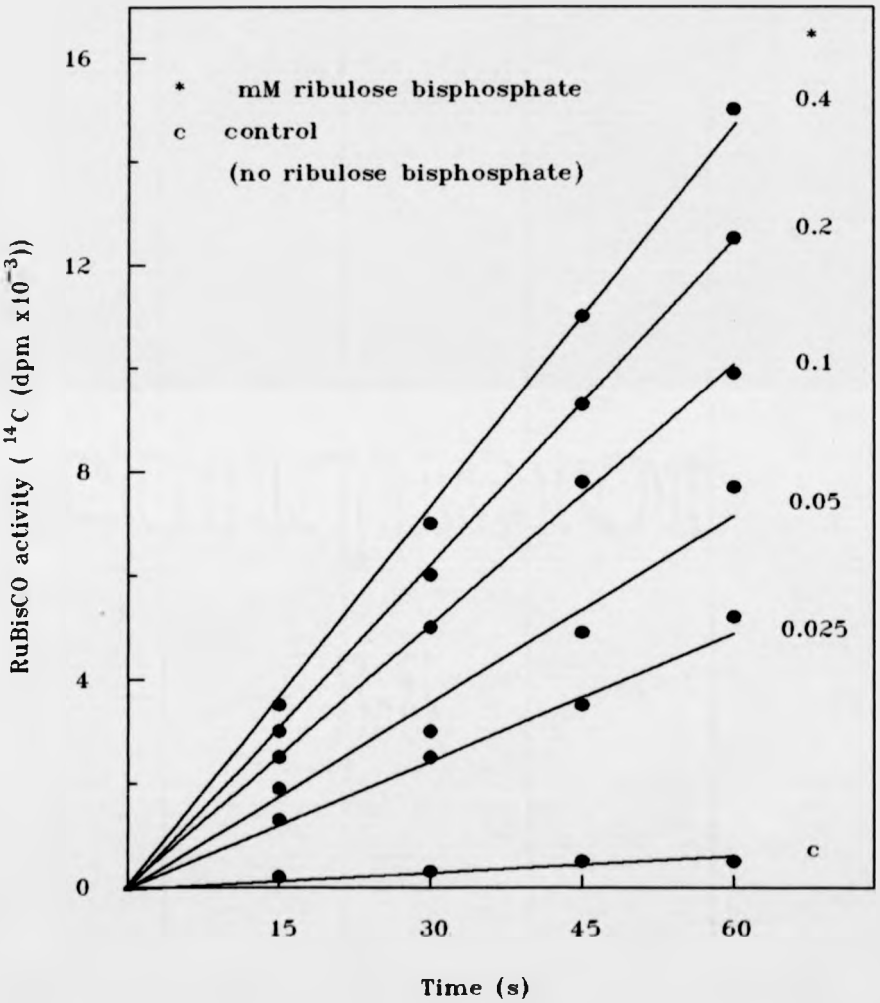
Partially purified RuBisCO from strain BC1 was incubated with a range of bicarbonate concentrations (0.4 to 5.6 mM) and a time course established for each.



The maximum rate for each bicarbonate concentration was converted to nmol CO_2 fixed/ min/ mg protein and plotted in figure 3.13.

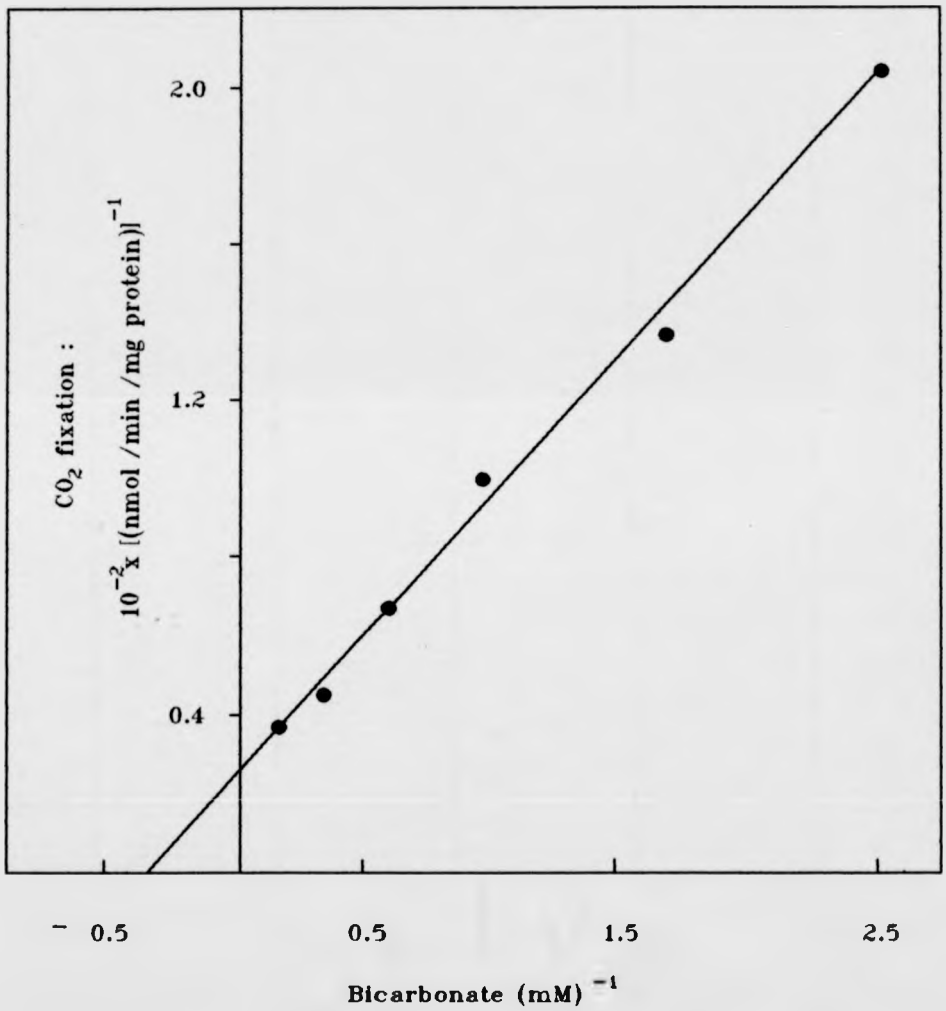
Figure 3.12 Strain BC1 RuBisCO K_m(RuBP) determination (time course experiments).

The partially purified RuBisCO from strain BC1 was incubated with a range of ribulose-1,5-bisphosphate concentrations (0.025 to 0.4 mM) and a time course established for each.



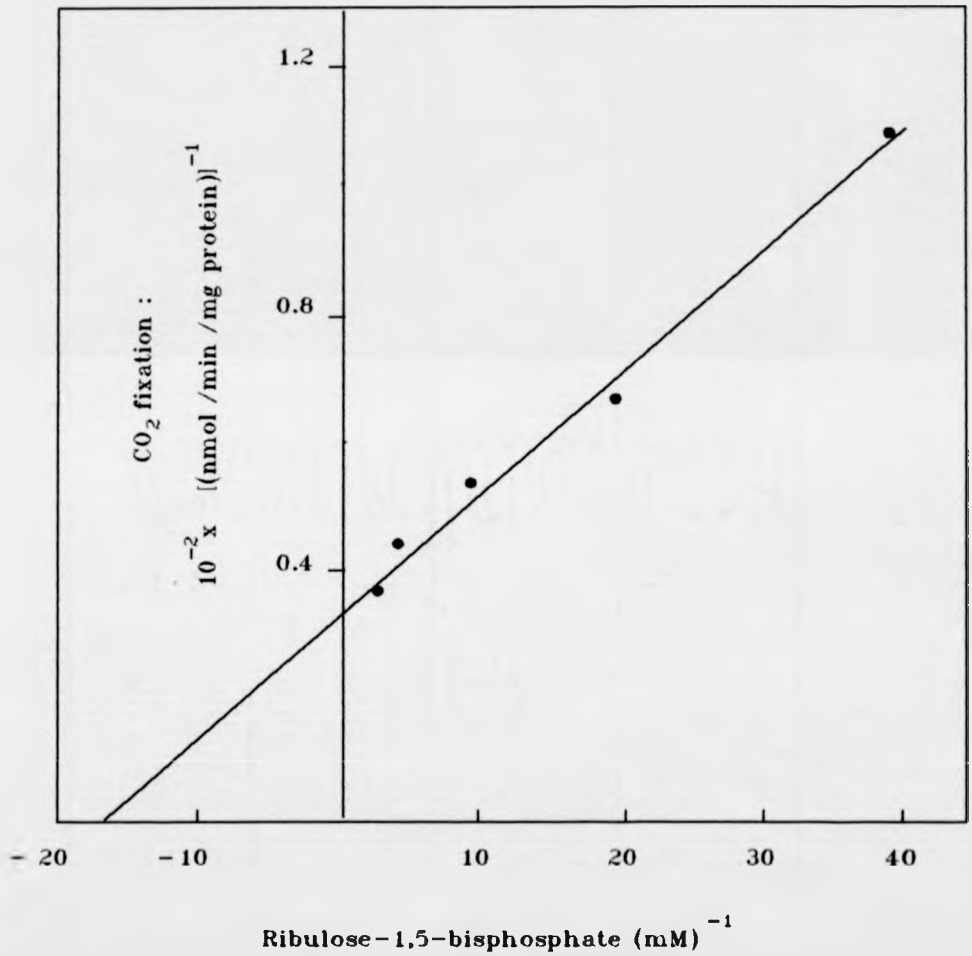
The maximum rate for each ribulose bisphosphate concentration was converted to nmol CO_2 fixed/ min/ mg protein and plotted in figure 3.14.

Figure 3.13 Strain BC1 RuBisCO $K_m(CO_2)$ determination. The maximal reaction rates derived from fig. 3.11 were plotted against the corrected bicarbonate concentrations The K_m(bicarbonate) was estimated from the Lineweaver-Burk plot below.



The apparent K_m(bicarbonate) was estimated from the intercept of the plotted line and the x-axis following linear regression analysis. This was converted to the apparent $K_m(CO_2)$.

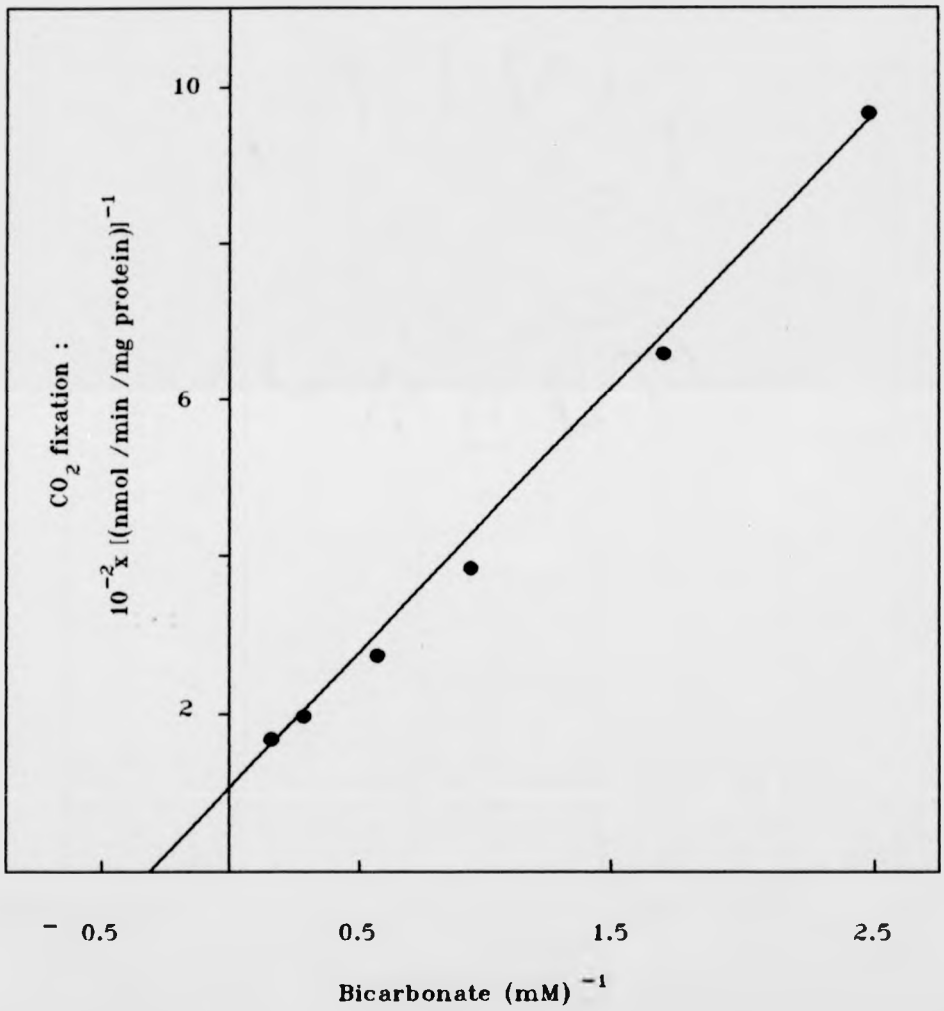
Figure 3.14 Strain BC1 RuBisCO K_m(RuBP) determination. The maximal enzyme rates derived from fig. 3.12 were plotted against the respective ribulose-1,5-bisphosphate concentrations.



The apparent K_m(ribulose-1,5-bisphosphate) was estimated from the intercept of the plotted line and the x-axis following linear regression analysis.

Figure 3.15 *T. ferrooxidans* RuBisCO $K_m(CO_2)$ determination.

The maximal enzyme rates for each bicarbonate concentration were plotted against the corrected substrate concentrations. The line of best fit was calculated by linear regression analysis.



The apparent K_m(bicarbonate) was converted to the apparent $K_m(CO_2)$.

Figure 3.16 *T. ferrooxidans* RuBisCO K_m(RuBP) determination.

The maximal reaction rates were plotted against each ribulose-1,5-bisphosphate concentration. The apparent K_m (ribulose-1,5-bisphosphate) was then calculated following linear regression analysis, as before.

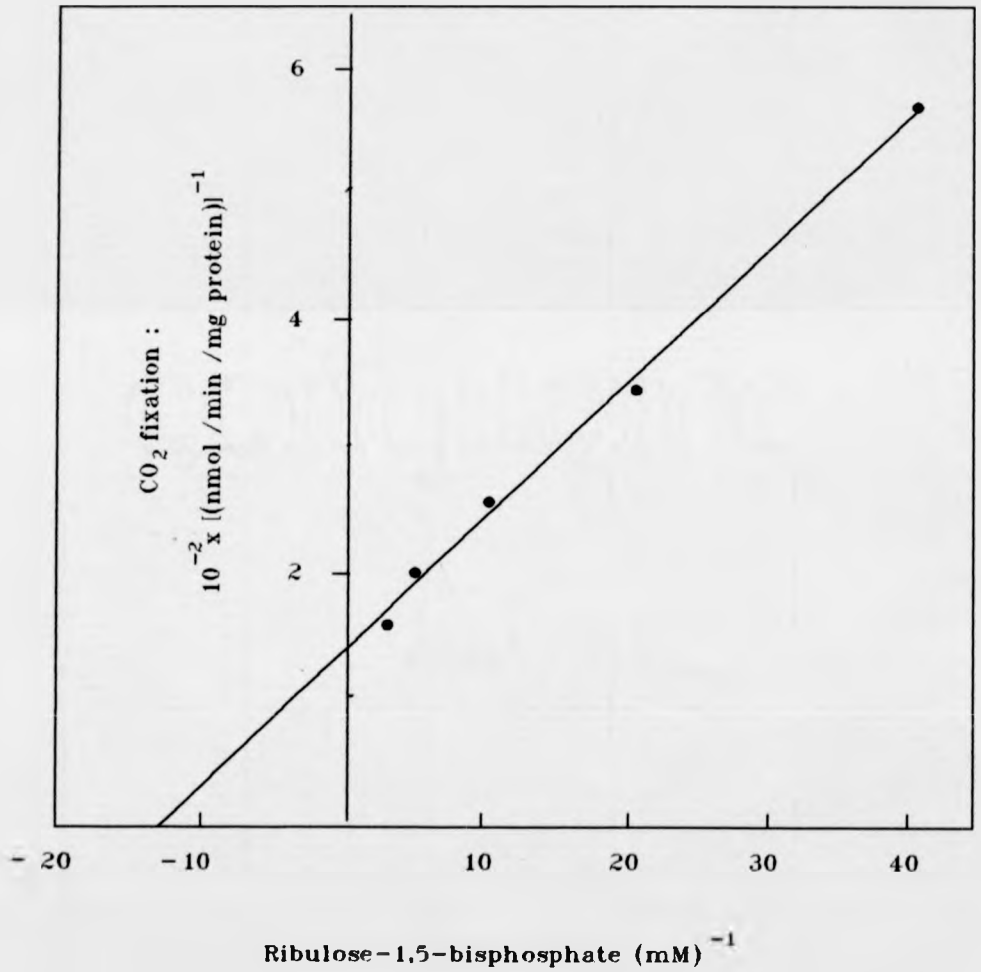
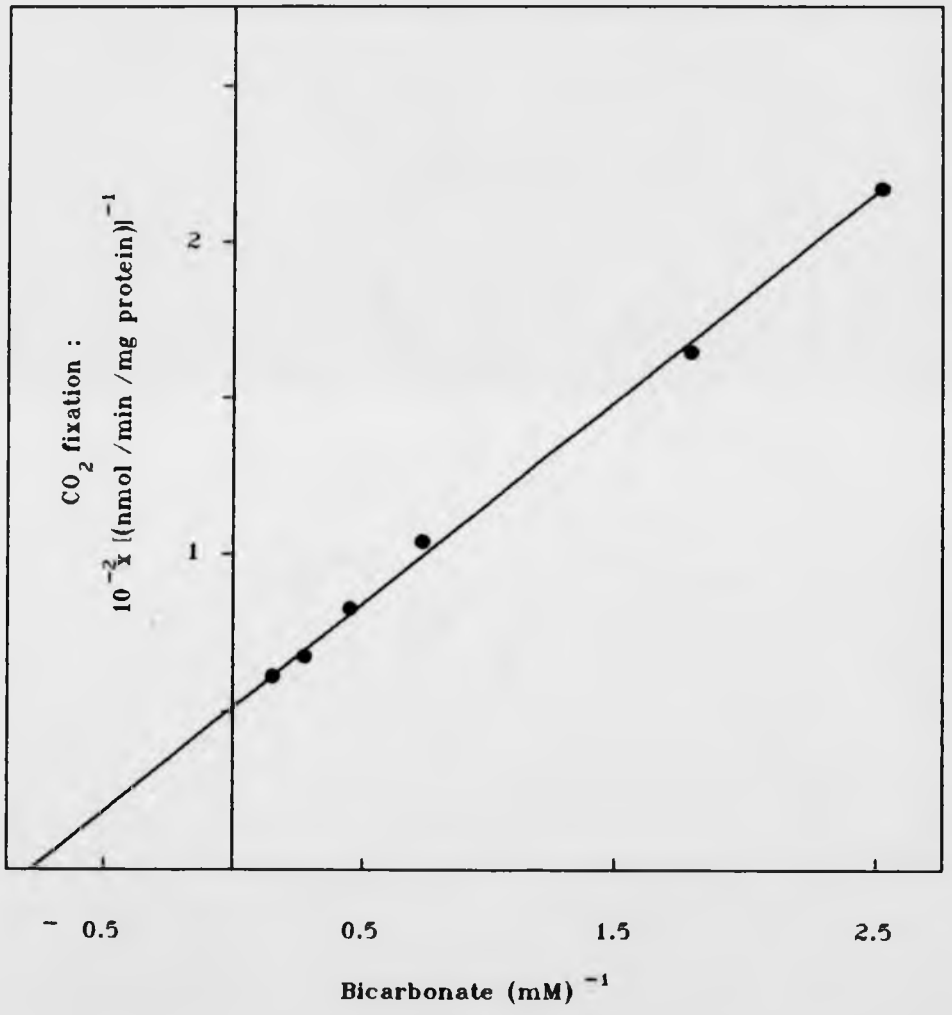


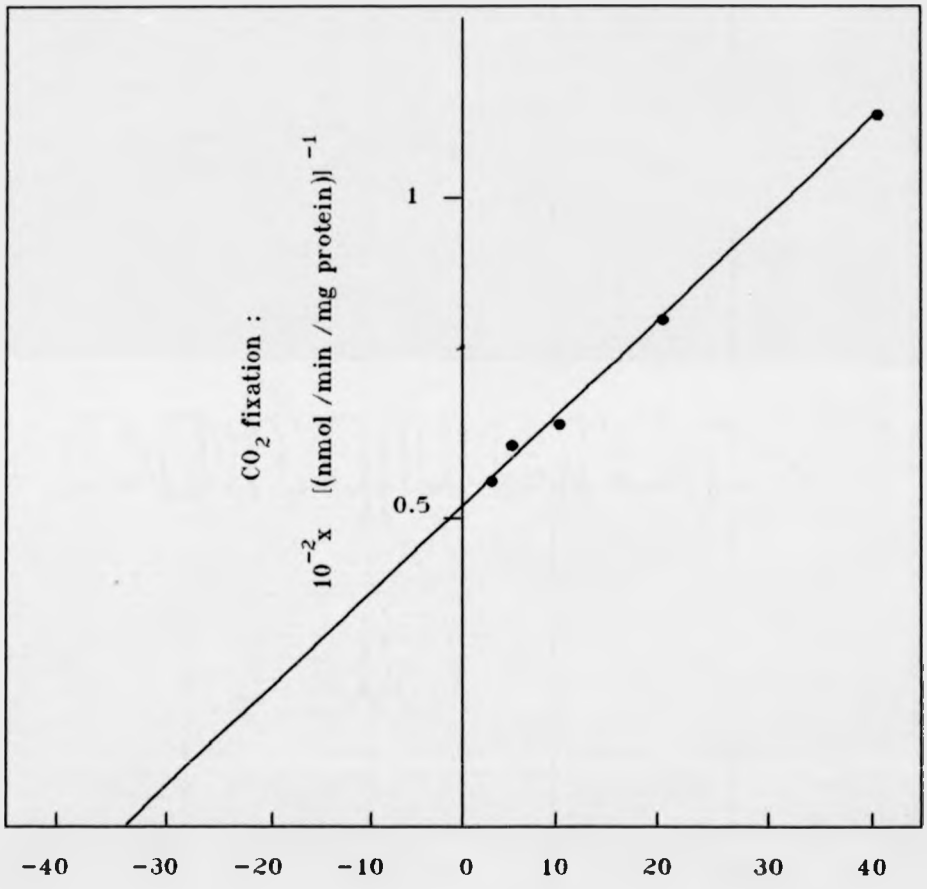
Figure 3.17 Pea RuBisCO $K_m(CO_2)$ determination.
The maximal reaction rate for each bicarbonate concentration was plotted against the corrected substrate concentrations. The K_m (bicarbonate) was calculated following linear regression analysis.



The apparent K_m (bicarbonate) was converted to the apparent $K_m(CO_2)$.

Figure 3.18 Pea RuBisCO K_m(RuBP) determination.

The maximal reaction rates were plotted against the respective ribulose-1,5-bisphosphate concentrations. The apparent K_m was determined following linear regression analysis, as before.



The RuBisCO substrate affinity data of strain BC1, *T. ferrooxidans* and *Pisum sativum* (illustrated in fig. 3.13 to 3.18) were also represented by means of Eadie-Hofstee plots (see table 3.5). The values obtained were very similar to, though generally slightly lower than, those attained by Lineweaver-Burk plots.

Table 3.5 Summary of RuBisCO substrate affinities.

	Apparent K_m	
	CO_2 (μM)	Ribulose-1,5-bisphosphate (μM)
Strain BC1 *	41 (42) 46	63 (57) 72
T. ferrooxidans *	54 (41) 40	83 (78) 88
Pea	23.5 (23)	30 (27)

Bracketed values were determined by Eadie-Hofstee plots.

* Duplicate results were derived from separate batches of cells.

3.7 Investigation for multiple forms of RuBisCO in strain BC1 and *T. ferrooxidans.*

In prokaryotes, RuBisCO enzymes with different structures, molecular weights and substrate affinities have been isolated (see table 1.2). Furthermore, the presence of two RuBisCO forms has been demonstrated in some anoxygenic photolithotrophic bacteria, although the physiological significance of this is uncertain (see Tabita, 1988).

However, relatively recent work by Hallenbeck *et al.* (1990a and 1990b) suggested that the presence of two RuBisCO forms in *Rhodobacter sphaeroides* represented more than various stages of this protein's evolution (McFadden, 1973). Following inactivation of either *cfxA* and/or *cfxB* (see section 1.3.4), Hallenbeck *et al.* (1990a) demonstrated that each form of RuBisCO played an essential role in cellular redox maintenance during photoheterotrophic growth at differing CO_2 concentrations. In addition, the two unlinked genetic regions encoding proteins involved in CO_2 fixation were shown to exert significant effect over the expression of each other.

The presence of multiple forms of RuBisCO was investigated in both strain BC1 and *T. ferrooxidans.* The control organism, *Rhodobacter sphaeroides*, which was known to possess two forms of RuBisCO (see table 1.2), was grown photoheterotrophically with butyrate as the electron donor (Gibson and Tabita, 1977a). Under such conditions the RuBisCO ratio (form I: form II) has been determined as approximately 0.5 (Jouanneau and Tabita, 1986). In contrast, the two enzymes are derepressed at different levels during autotrophic growth, with an observed ratio of approximately 2 (Jouanneau and Tabita, 1986).

From the evidence already provided by native PAGE (fig. 3.10), western blotting with anti-form I RuBisCO (fig. 3.3) and comparison of substrate affinity data (table 3.5) with table 1.2, it seemed most likely

that the predominant RuBisCO protein from both strain BC1 and *T. ferrooxidans* was of the form I type. The growth conditions for these two chemolithotrophic bacteria were therefore chosen such that they might favour the expression of a form II type RuBisCO with low CO_2 affinity, should one exist, i.e. CO_2 supplied in excess with an alternate, heterotrophic carbon source.

Consequently, the nutritionally versatile moderate thermophile, strain BC1, was grown mixotrophically in the presence of 1% (v/v) CO_2, 50 mM ferrous iron and 1 mM glucose as an alternative carbon source. Under similar conditions up to 25% of the total carbon assimilated has been demonstrated to have arisen from RuBisCO-dependent CO_2 fixation, the oxidative pentose phosphate pathway having provided the remainder (Wood and Kelly, 1985).

The obligate autotroph *T. ferrooxidans* was grown in the presence of 50 mM ferrous iron and gassed with 1% (v/v) CO_2.

The method employed for this investigation was essentially that developed by Sani (1985), for the partial purification of RuBisCO from a number of the *Rhodospirillaceae*. Its major advantage was a short sucrose gradient centrifugation stage which resulted from the use of a fixed angle (2.5 hours) as opposed to a swing-out rotor (18 hours). This was especially important for dealing with unstable RuBisCOs.

Soluble protein extract from all three bacterial sources was centrifuged in separate 0.2 to 0.8 M sucrose step gradients. Following a brief settling period, 1 ml fractions were collected from the bottom and assayed for both RuBisCO activity and protein. The two sets of data were then superimposed in fig. 3.19a for strain BC1, fig. 3.20a for *T. ferrooxidans* and fig. 3.21a for *R. sphaeroides*.

The most active RuBisCO fractions were pooled and then screened for the presence of one or more different molecular forms of this enzyme by the polyacrylamide gel assay.

One distinct peak of enzyme activity was observed for strain BC1 (fig. 3.21b) which represented the presence of one RuBisCO form.

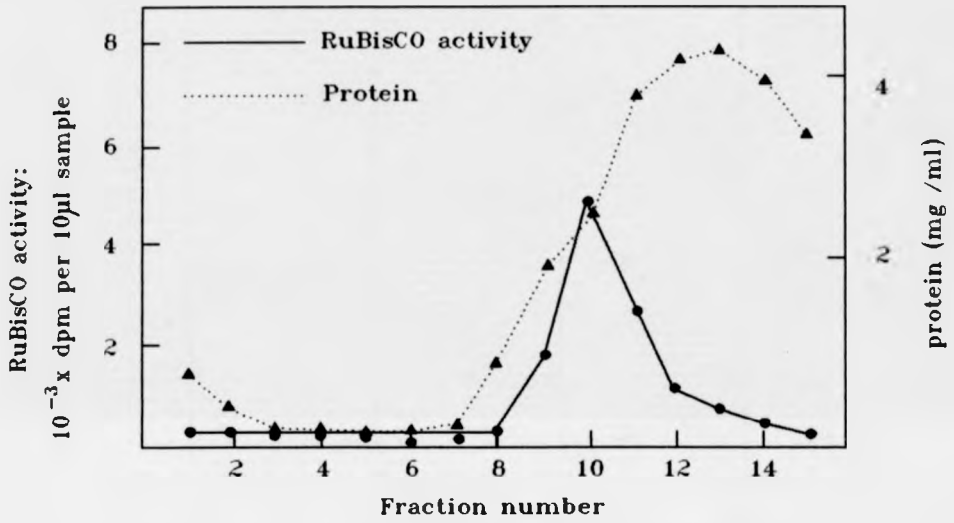
Following electrophoresis, one peak of RuBisCO activity was also detected by the polyacrylamide gel assay for *T. ferrooxidans* (see fig. 3.20b). This corresponded to slice number 22 and was very similar to that obtained for strain BC1 (slice number 20). Previous work by Shively *et al.* (1989) provided similar evidence for a single, form I type RuBisCO in *T. ferrooxidans* following sucrose density centrifugation. However, the use of RuBisCO large sub-unit DNA probes from *Anacystis nidulans* and *Rhodospirillum rubrum*, in the same report, suggested the presence of two RuBisCO forms.

Finally, two peaks of RuBisCO activity corresponding to the two distinct forms of this enzyme (Gibson and Tabita, 1977a) were observed for *R. sphaeroides* following the polyacrylamide gel assay (fig. 3.21b). The peak corresponding to the form I type RuBisCO in this organism migrated to a very similar location (slice number 20, fig. 3.21b) as the RuBisCOs from both chemolithotrophic sources.
By contrast, the gel assay activity associated with the form II type RuBisCO from *R. sphaeroides* was distinct and well separated from the form I type.

It is accepted that the migration of proteins down a polyacrylamide gel is subject to factors other than molecular weight alone, such as charge and size. However, co-migration of the RuBisCO from strain BC1 and *T. ferrooxidans* with those from spinach (fig. 3.10) and

Figure 3.19 RuBisCO tube gel assays for strain BC1.

a) Soluble protein extract from mixotrophically-grown (glucose+CO_2) strain BC1 was centrifuged in a 0.2 to 0.8M sucrose step gradient. 1 ml fractions were assayed for RuBisCO activity and protein.



b) Following gel electrophoresis of pooled, active RuBisCO fractions, 1 mm slices were assayed for activity.

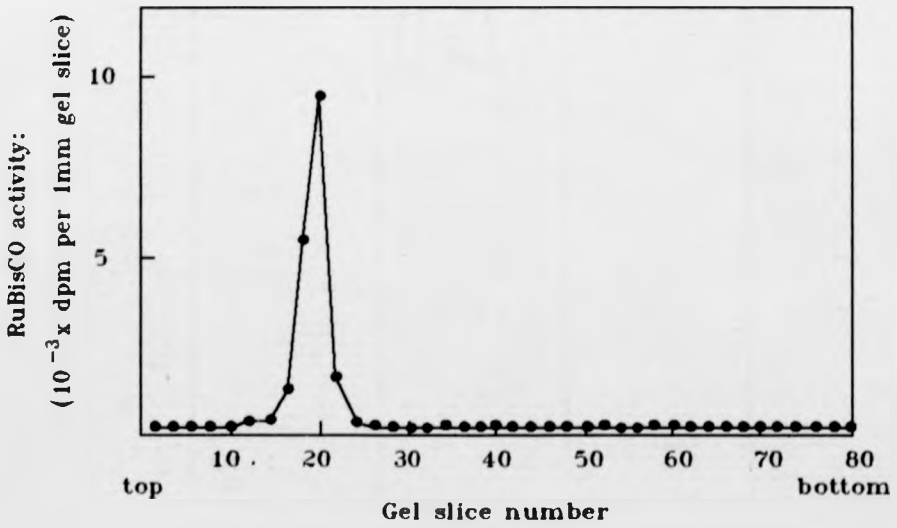
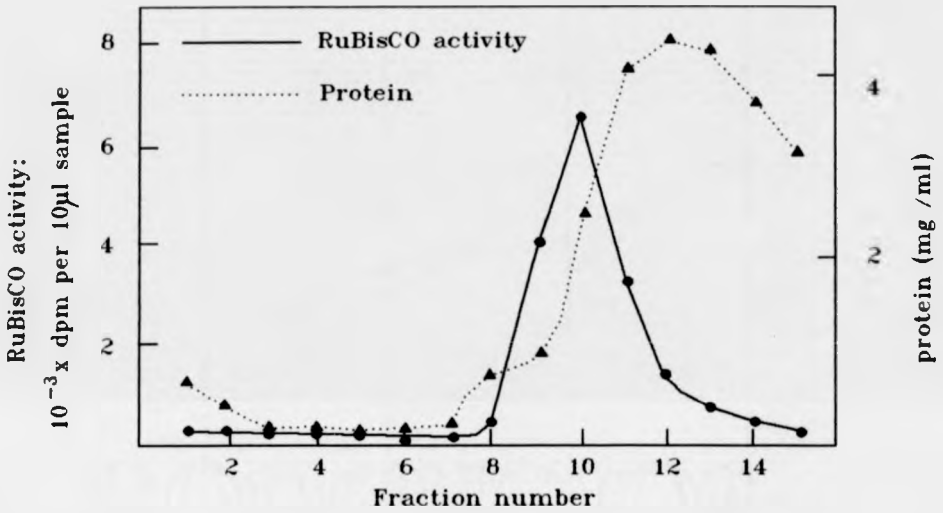


Figure 3.20 RuBisCO tube gel assays for *Thiobacillus ferrooxidans*

a) Soluble protein extract from autotrophically-grown (1% CO_2) *Thiobacillus ferrooxidans* was centrifuged in a 0.2 to 0.8 M sucrose step gradient. 1 ml fractions were then assayed for RuBisCO activity and protein.



b) Following gel electrophoresis of pooled, active RuBisCO fractions, 1 mm slices were assayed for activity.

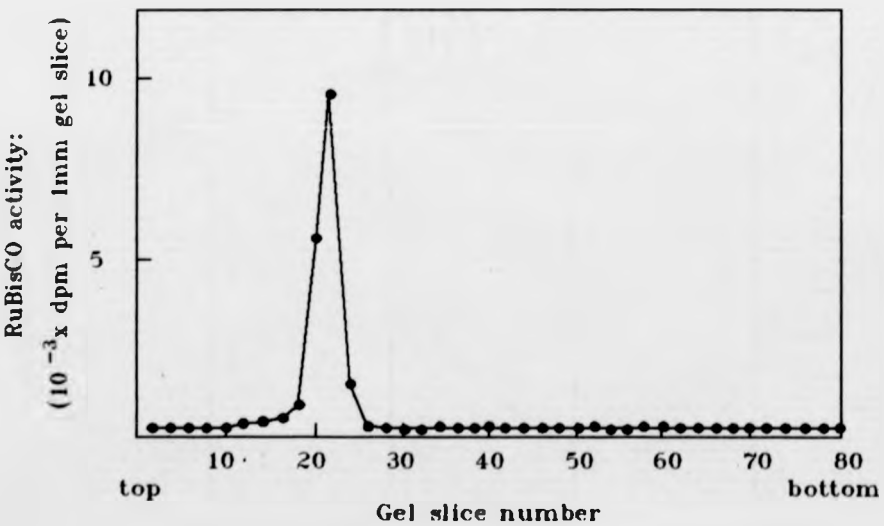
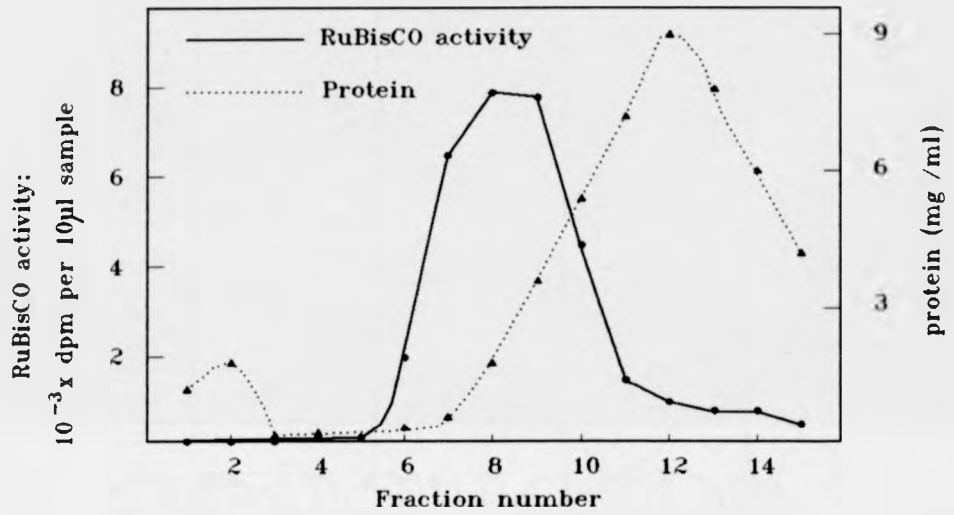
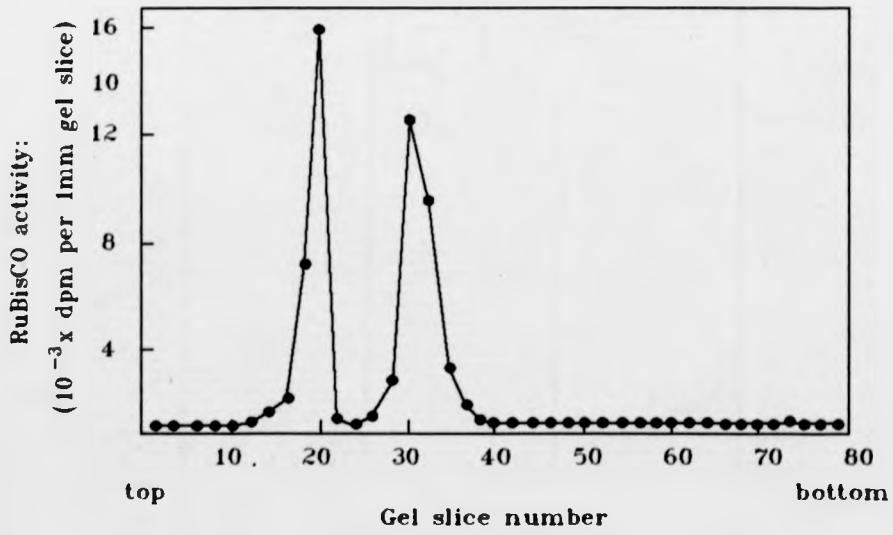


Figure 3.21 RuBisCO tube gel assay for *Rhodobacter sphaeroides*

a) Soluble protein extract from photoheterotrophically-grown (butyrate-bicarbonate) *Rhodobacter sphaeroides* was centrifuged in a 0.2 to 0.8M sucrose step gradient. 1ml fractions were assayed for RuBisCO activity and protein.



b) Following gel electrophoresis of pooled, active RuBisCO fractions, 1 mm slices were assayed for activity.



R. sphaeroides, form I, (fig. 3.19 to 3.21), in conjunction with their determined substrate affinities (table 3.5) and anti-form I RuBisCO western blot (fig. 3.3) strongly suggested that both were of the form I (L_8S_8) type.

CHAPTER 4

CO_2 uptake

4.1 Introduction

A wide variety of photosynthetic micro-organisms have the ability to accumulate intracellular C_i by means of inducible, high affinity carbon concentrating mechanisms (CCMs), see section 1.4.

The cyanobacteria are known to possess a form I type RuBisCO that exhibits an unusually poor substrate affinity for CO_2 (table 1.2). In fact at air levels of saturation, and in the presence of O_2, its effective $K_m(CO_2)$ is about 600 μM (Pierce and Omata, 1988). However, the presence of a high affinity CCM allows these organisms to compensate for the observed difference between the CO_2 concentration in their environment and that required for effective RuBisCO activity. Intracellular accumulation levels of up to 1000-times the external C_i concentration are not uncommon for these organisms (Kaplan *et al.*, 1991).

In contrast to the wealth of data available for the photosynthetic bacteria, an inducible, high affinity CCM has only been demonstrated in one chemolithotrophic bacterium, *Thiobacillus neapolitanus* (Holthuijzen *et al.*, 1987).

During autotrophic growth with air levels of CO_2, this organism responds by derepressing its RuBisCO synthesis three- to five-fold. However, the low partial pressure of atmospheric CO_2 and its associated slow diffusion through water conspire to lower intrinsic rates of delivery of CO_2 to this organism. Consequently, although *T. neapolitanus* possesses the typical form I type RuBisCO with a high CO_2 affinity (see table 1.2), it also responds to intracellular CO_2 limitation by the induction of a high affinity CCM (Holthuijzen *et al.*, 1987).

CO_2 transport has therefore been studied comparatively in the moderate thermophiles and *T. ferrooxidans*, the latter serving as a control for the thermophile, as noted previously.

4.2 CO_2 transport with resuspended cells

All cells (*T. ferrooxidans*, strain BC1 and strain ALV) were grown autotrophically to mid-log phase in batch culture with 1% (v/v) CO_2 in air. The washed cell suspensions were then allowed to equilibrate back to their respective growth temperatures (30°C for *T. ferrooxidans*, thermophiles at 45°C) prior to the addition of ferrous iron as the energy source to support transport processes.

Following the addition of ^{14}C-labelled bicarbonate, radioactive label incorporation into the cells was followed over a assay period of 5 minutes (fig. 4.1).

Although bicarbonate was the source of C_i added, because of the pH of the assay (pH 1.6), the predominant C_i species present was CO_2 (greater than 99.9%). Consequently, it was more appropriate to express the final concentration of added label as a percentage (v/v) of CO_2 in air than as mM bicarbonate.

A final assay concentration of 0.1% (v/v) CO_2 was chosen since this was the lowest concentration required for saturation of the transport system studied in strain BC1 (fig. 4.6).

Following resuspension, *T. ferrooxidans* (+ 50 mM ferrous iron) rapidly took up label at a rate corresponding to approximately 50 nmol CO_2/ min/ mg protein (fig. 4.1). Similar observations were made with air-grown *T. ferrooxidans* cells, although CO_2 was transported at a slightly faster rate of 70 nmol CO_2/ min/ mg protein in the presence of 50 mM ferrous iron (results not illustrated). Comparable

transport rates have been reported for the obligate chemolithotroph *T. neapolitanus* (Holthuijzen *et al.*, 1987) and resulted in high intracellular accumulation ratios of C_i (500- to 800-fold for excess CO_2-grown cells and 1000- to 1500-fold for cells grown under C_i limitation).

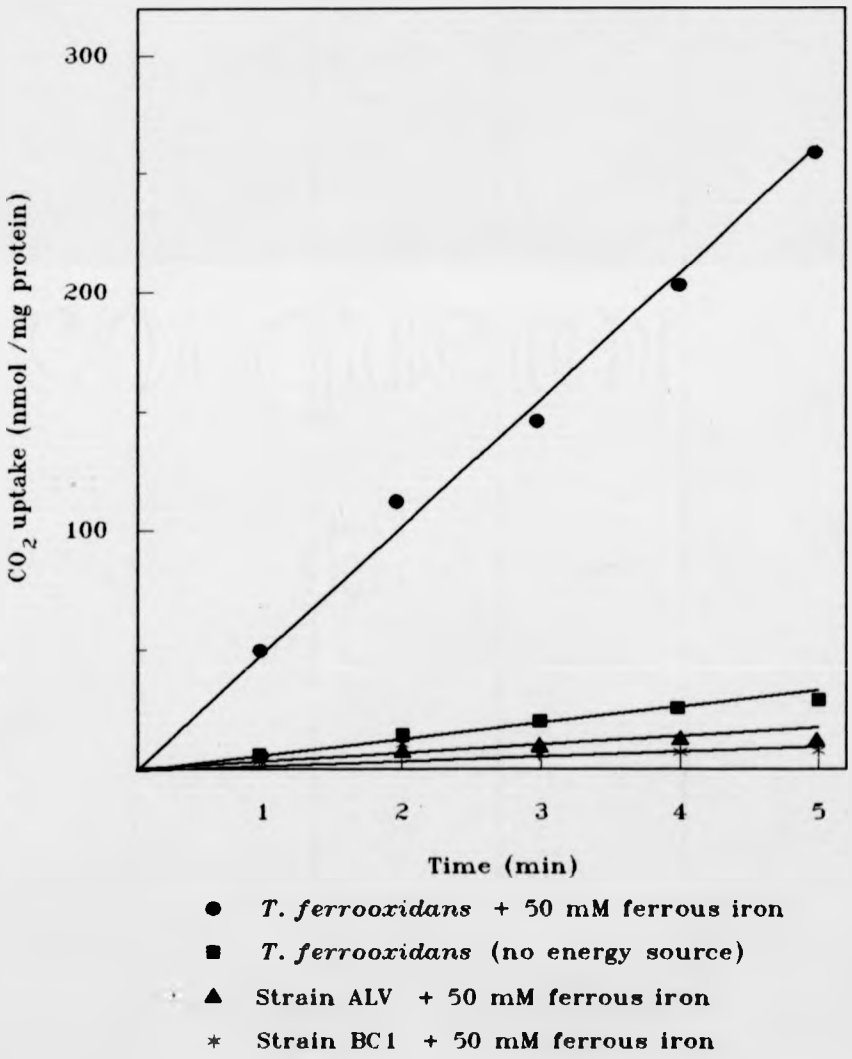
However, when the exogenous energy source was omitted with *T. ferrooxidans* (fig. 4.1), the associated CO_2 transport rate was reduced ten-fold. The energy dependence of $^{14}CO_2$ accumulation clearly indicated the presence of a transport system as opposed to the process of diffusion. These results were also consistent with those obtained with *T. neapolitanus* (Holthuijzen *et al.*, 1987), in which C_i transport was only observed in energized cells, i.e. in the presence of the electron donor, thiosulphate.

In contrast, only very low CO_2 transport rates were detected for both of the moderate thermophile strains BC1 and ALV. Furthermore, the presence or absence of ferrous iron had no effect on the CO_2 uptake rate for either of these organisms (only CO_2 transport with ferrous iron is illustrated in fig. 4.1), which remained at less than 5 nmol CO_2/ min/ mg protein. Tetrathionate was not added to strain BC1 (fig. 4.1) since this was only required to satisfy this organism's sulphur-source requirement during growth. However, identical CO_2 transport rates were observed when tetrathionate was present (results not illustrated).

Several factors suggested that the uptake rates obtained for the moderate thermophiles were not representative of the *in vivo* state of the cells.

Both *T. ferrooxidans* and strain BC1 have demonstrated almost identical growth rates (fig. 3.1 and 3.2) and CO_2 fixation capacities (tables 3.2 and 3.3) when grown autotrophically with 1% (v/v) CO_2 in

Figure 4.1 CO_2 uptake by cell suspensions of the moderate thermophiles and *T. ferrooxidans*. All cells were grown autotrophically with 1% (v/v) CO_2 in air. Following harvesting, cells were resuspended to $O.D_{440}$ 0.15 and added to assay flasks at the appropriate temperature. Ferrous iron (where indicated) was added, the flasks sealed, and labelled bicarbonate injected. The final assay concentration was 0.1% (v/v) CO_2.



air. Such conditions were assumed to be non-limiting with respect to carbon, since an increase in the gas supply to 5% (v/v) CO_2 in air had no effect on the growth rate of either organism (results not illustrated). Consequently, one would have expected both organisms to exhibit similar CO_2 transport rates from an atmospheric concentration of ^{14}C-labelled CO_2 that would be non-limiting to growth. Identical observations were made with assay concentrations of 0.1% and 1% (v/v) $^{14}CO_2$ (results not illustrated).

The above evidence suggested that the process of cell preparation had in some way severely reduced the CO_2-uptake capacity of the two moderate thermophiles. Harvesting by filtration produced identical results (not illustrated), indicating that the problem was not restricted to centrifugation of the cells.

4.3 "Regeneration" of resuspended cells

The extremely low transport efficiences exhibited by the moderate thermophiles, following harvesting and resuspension, were further investigated in strain BC1 and strain ALV (fig. 4.2).

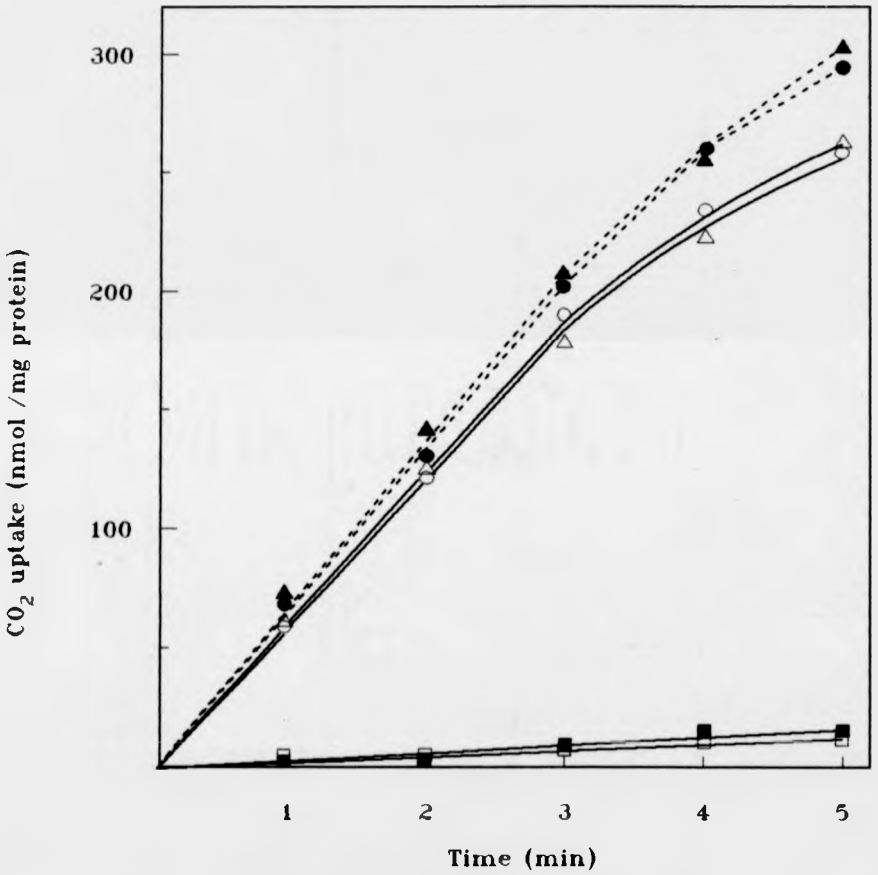
Washed cell suspensions were incubated at 45°C with regular additions of ferrous iron over a period of between 2 and 6 hours.

Following regeneration for a 2 hour period both organisms incorporated label at the previously observed (fig. 4.1) rate of less than 5 nmol CO_2/ min/ mg protein.

However, following a 4 hour or longer period, $^{14}CO_2$ was rapidly incorporated into both organisms at a rate of 50 to 60 nmol CO_2/ min/ mg protein. This was comparable to the rate obtained with energized *T. ferrooxidans* cells (fig. 4.1).

Of the two moderate thermophiles investigated, strain ALV appeared to consistently exhibit very slightly higher CO_2 transport rates than strain BC1.

Figure 4.2 The necessity for "regeneration" prior to CO_2 uptake by the moderate thermophiles. All cells were grown autotrophically with 1% (v/v) CO_2 in air. Harvesting and resuspension were as in fig. 4.1. Strains BC1 and ALV were then "regenerated" by regular additions of ferrous iron over a range of 2 to 6 hours. All cells were assayed as in fig. 4.1.



open symbols represent strain BC1

closed symbols represent strain ALV

□ ■ following a 2 hour regeneration period

○ ● following a 4 hour regeneration period

△ ▲ following a 6 hour regeneration period

A standard regeneration period of 4 hours was subsequently adopted for the moderate thermophiles, since this was the shortest time required for restoration of maximal CO_2 transport in these organisms.

This regeneration period was investigated in further detail with strain BC1 (fig. 4.3).

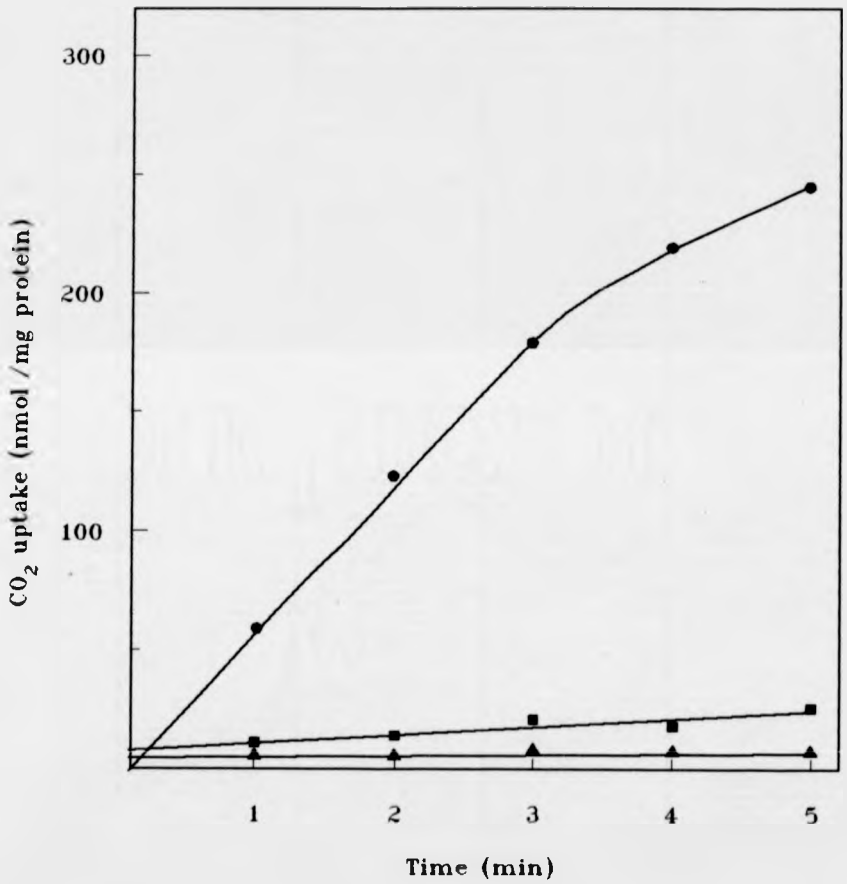
Radioactive label was rapidly taken up and accumulated by strain BC1 following the control regeneration of 4 hours.

In contrast, when ferrous iron had been omitted during this period, regeneration was no longer possible and consequently no appreciable uptake of $^{14}CO_2$ was observed. This clearly indicated that the process of regeneration was energy-dependent. It may therefore be possible that the process of harvesting had dissipated some component essential for transport, such as the proton motive force (pmf) or one of its constituents, the pH gradient or the electric potential. The latter of these two has been demonstrated to be the major driving force for uptake of C_i in *T. neapolitanus* (Holthuijzen *et al.*, 1987).

The final assay in fig. 4.3 included not only ferrous iron in the regeneration, but also chloramphenicol which would inhibit all *de novo* protein synthesis. Norris (pers. comm.) had previously demonstrated that 2 µg/ml of this antibiotic was sufficient to inhibit the growth of strain BC1.

From fig. 4.3 it was clear that chloramphenicol also prevented "regeneration" of this organism. The implication of this observation was that harvesting had damaged more than just the pmf, or one of its components, and that protein synthesis was essential before optimal CO_2 transport could be achieved. Furthermore, had the regeneration period simply been necessary for re-energization of the transport system, then one would have expected almost spontaneous uptake of labelled CO_2 following addition of the energy source, ferrous iron.

Figure 4.3 Effect of chloramphenicol on CO_2 uptake by strain BC1. Cells were grown autotrophically with 1% (v/v) CO_2 in air. Harvesting and resuspension were as in fig. 4.1. The regeneration period was then investigated further by the omission of ferrous iron or inclusion of chloramphenicol. All assays were as in fig. 4.1.



● following a 4 hour regeneration period plus iron

▲ following a 4 hour regeneration period in which ferrous iron had been omitted

■ following a 4 hour regeneration period plus iron, in the presence of chloramphenicol (2 μg/ml)

However, it is not known whether biosynthesis was necessary to replace a specific protein component of the CO_2 transport system, or some component essential for driving the uptake system, such as ATP sythesis.

Further attempts were made to determine whether "regeneration" was a specific requirement for CO_2 transport by the consideration of another transport system. Cells were harvested and resuspended in the usual manner and ^{35}S-labelled methionine then added. No incorporation of label was achieved over a range of assay periods (0 to 60 seconds through to 0 to 60 minutes). However, the same situation was apparent when cells were used that had been removed directly from culture flasks without centrifugation or filtration. The most likely explanation for this was that strain BC1 had only a very small methionine pool size or that methionine could not be transported.

An alternative system, possibly for uptake of essential ions such as potassium, still needs to be studied to clarify the question of an energized cell state with respect to transport processes.

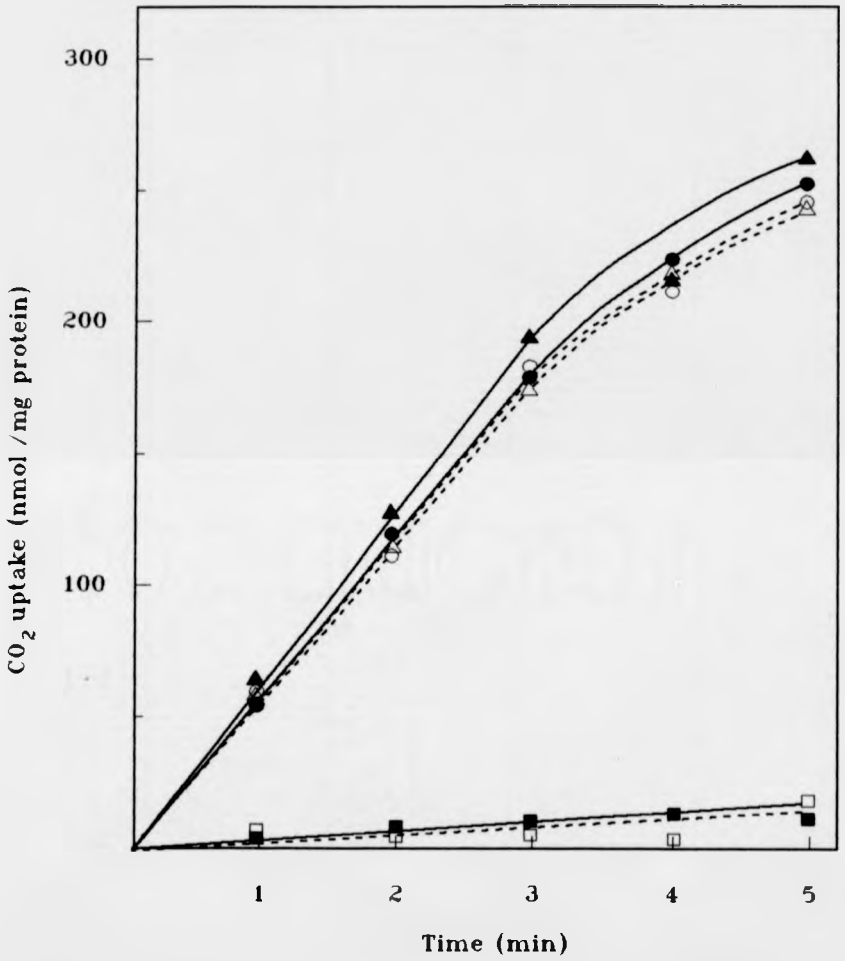
4.4 Comparison of CO_2 transport by growing and "regenerated" cells

The necessity for regeneration of the moderate thermophiles prior to CO_2 transport studies was both time consuming and inconvenient. Consequently, it was desirable to use growing cells that had not been harvested by either centrifugation of filtration.

The CO_2 uptake capacity of resuspended, regenerated and growing cells was compared (fig. 4.4).

Following addition of ^{14}C-bicarbonate, both regenerated and growing cells rapidly incorporated label at almost identical rates of approximately 50 nmol CO_2/ min/ mg protein. This clearly indicated that either of the methods employed were suitable for measuring CO_2 transport.

Figure 4.4 A comparison of CO_2 uptake by strain BC1; growing cells and cell resuspensions. Autotrophically and chemolithoheterotrophically grown cells were compared. All assays were as in fig. 4.1.



solid symbols: chemolithoheterotrophically grown cells (50 mM ferrous iron + 0.02% (w/v) yeast extract)

open symbols: autotrophically grown cells (1% (v/v) CO_2)

▲ △ growing cells

● ○ resuspended cells with 4 hour regeneration

■ □ resuspended cells without 4 hour regeneration

By comparison, the control incubations of resuspended but non-regenerated cells took up label at the characteristically lower rate of less than 5 nmol CO_2/ min/ mg protein. This also confirmed that the process of cell harvesting had been responsible for the almost complete inactivation of the CO_2 transport system in the moderate thermophiles.

Finally, from fig. 4.4 it was apparent that chemolitho-heterotrophically grown strain BC1 cells were capable of CO_2 transport at an equal rate to autotrophically grown cells. Under such growth conditions strain BC1 exerts complete repression of RuBisCO, as detectable by SDS-PAGE (Norris, 1989b), and obtains approximately 95% of its cellular carbon requirement from the yeast extract (Wood and Kelly, 1983). This suggested that the CO_2 transport system present was constitutively expressed and not therefore directly linked to the autotrophic growth capacity of strain BC1.

4.5 Investigation for the presence of inducible, high-affinity CO_2 transport systems in strain BC1 and *T. ferrooxidans*.

The CO_2 transport experiments that had been performed previously involved assay conditions in which the $^{14}CO_2$ concentration had been in excess. However, the most important question to address with strain BC1 and *T. ferrooxidans* was whether both organisms were capable of similar CO_2 transport rates during growth with air levels of CO_2.

Consequently, both organisms were grown autotrophically in 1 litre reactors with air and 1% (v/v) CO_2 in air (as in fig. 3.1 and 3.2). Each batch of cells was then assayed over a time course of 5 minutes with a range of $^{14}CO_2$ concentrations (0.02% to 0.23% (v/v) CO_2). The time course results are illustrated only for strain BC1 (fig. 4.5.1 and 4.5.2).

In order to alleviate the requirement for regeneration of the moderate thermophile, growing cells were used in preference to those that had been centrifuged or filtered.

A time course was subsequently established for each CO_2 concentration (see fig. 4.5.1 for air-grown strain BC1; fig. 4.5.2 for 1% (v/v) CO_2 in air-grown strain BC1) and the maximal transport rates converted to nmol CO_2/ min/ mg protein.

The calculated rates for both growth conditions, and for each organism, were then plotted against their respective CO_2 assay concentrations (see fig. 4.6 for strain BC1, fig. 4.7 for *T. ferrooxidans*).

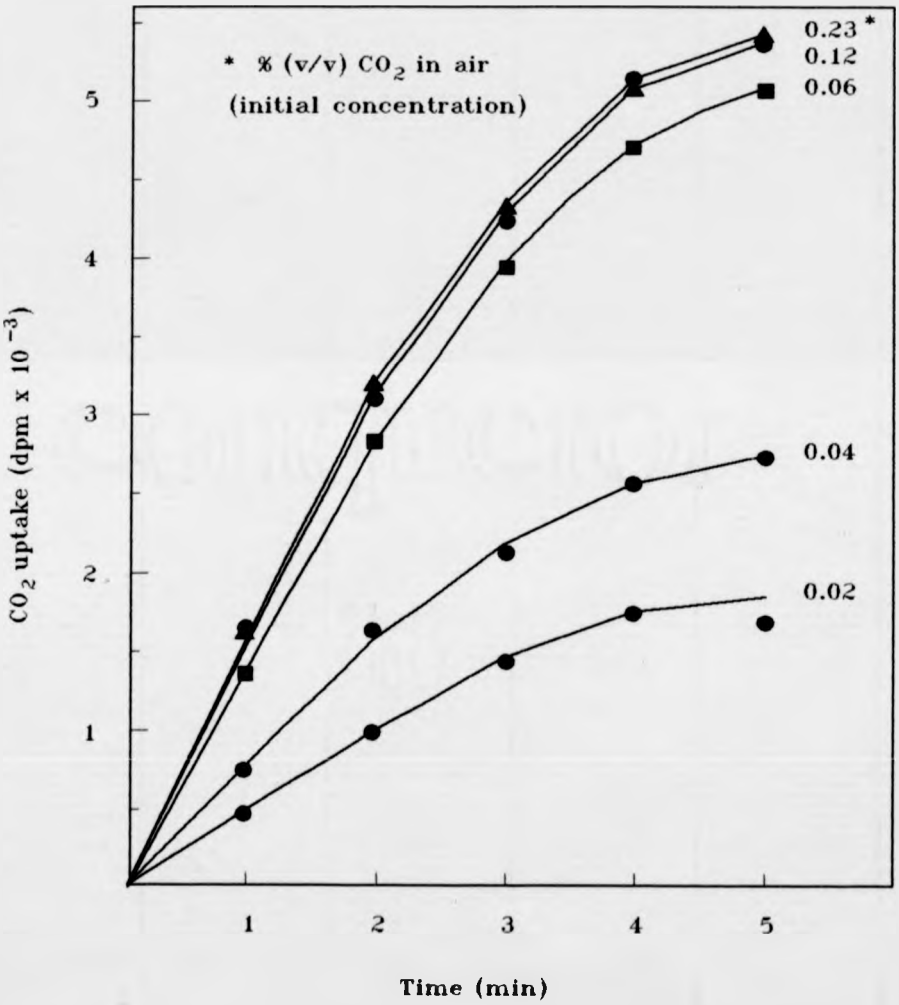
Strain BC1 (fig. 4.6) demonstrated a maximum CO_2 transport rate of 70 nmol CO_2/ min/ mg protein for autotrophically, air-grown cells. This was very similar to that of 65 nmol CO_2/ min/ mg protein obtained with cells grown in the presence of 1% (v/v) CO_2 in air.

A concentration of 0.03% (v/v) CO_2 in air supported transport rates reduced by approximately 50%. Under such conditions, cells that had been pre-cultured with either CO_2 concentration showed similar CO_2 uptake rates of between 30 and 35 nmol CO_2/ min/ mg protein.

The similarity of transport rates shown by strain BC1, whether grown with air or 1% (v/v) CO_2 in air, demonstrated that this organism was not capable of inducing a high affinity CO_2 transport system during autotrophic growth with limiting CO_2.

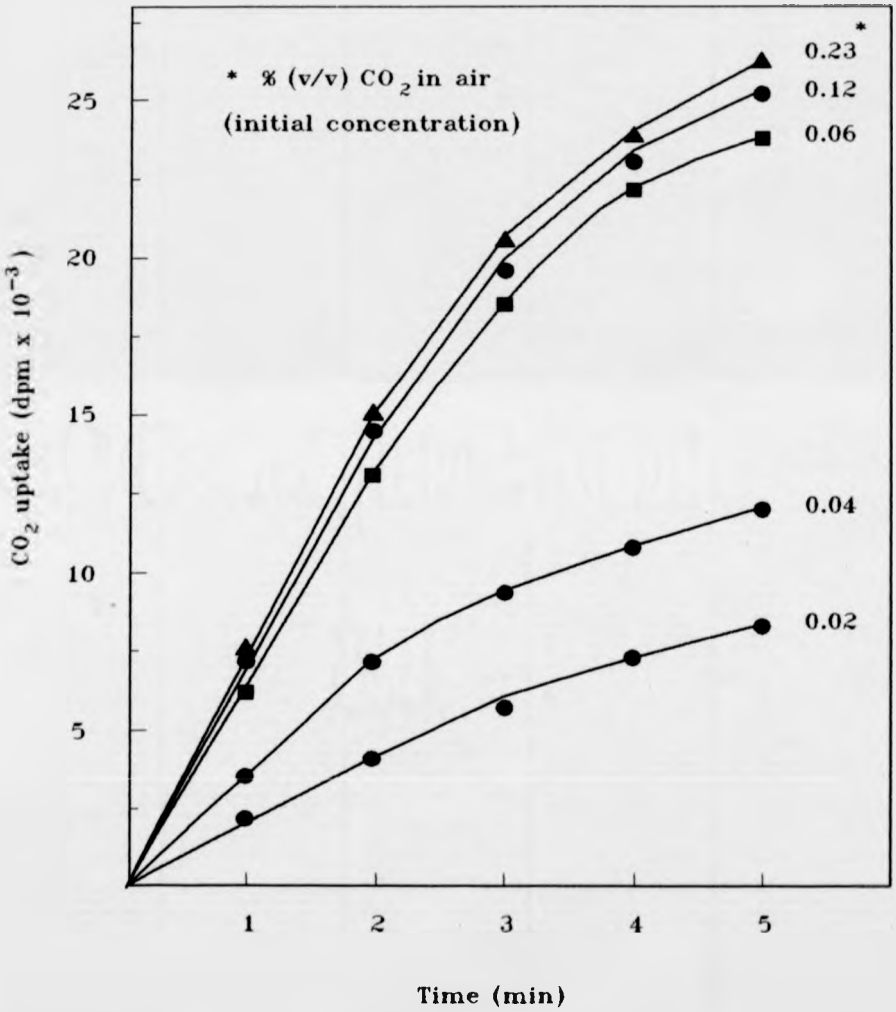
The constitutive system present in strain BC1 was clearly of low affinity, especially when compared with the inducible systems present in many cyanobacteria (see Miller, 1990; Coleman, 1991), and required 0.1% (v/v) CO_2 in air to achieve saturation. This concentration of CO_2 had previously been demonstrated to allow maximal autotrophic iron-oxidation rates by strain BC1 in 1 litre reactors when compared with concentrations of 1% and 5% (v/v) CO_2 in air (results not illustrated,

Figure 4.5.1 Effect of CO_2 concentration on its uptake by strain BC1 grown with air (time course experiments). Following de-gassing of the medium with CO_2-free air, a range of CO_2 concentrations (0.02 to 0.23% (v/v)) were established. A time-course was then determined for each concentration.



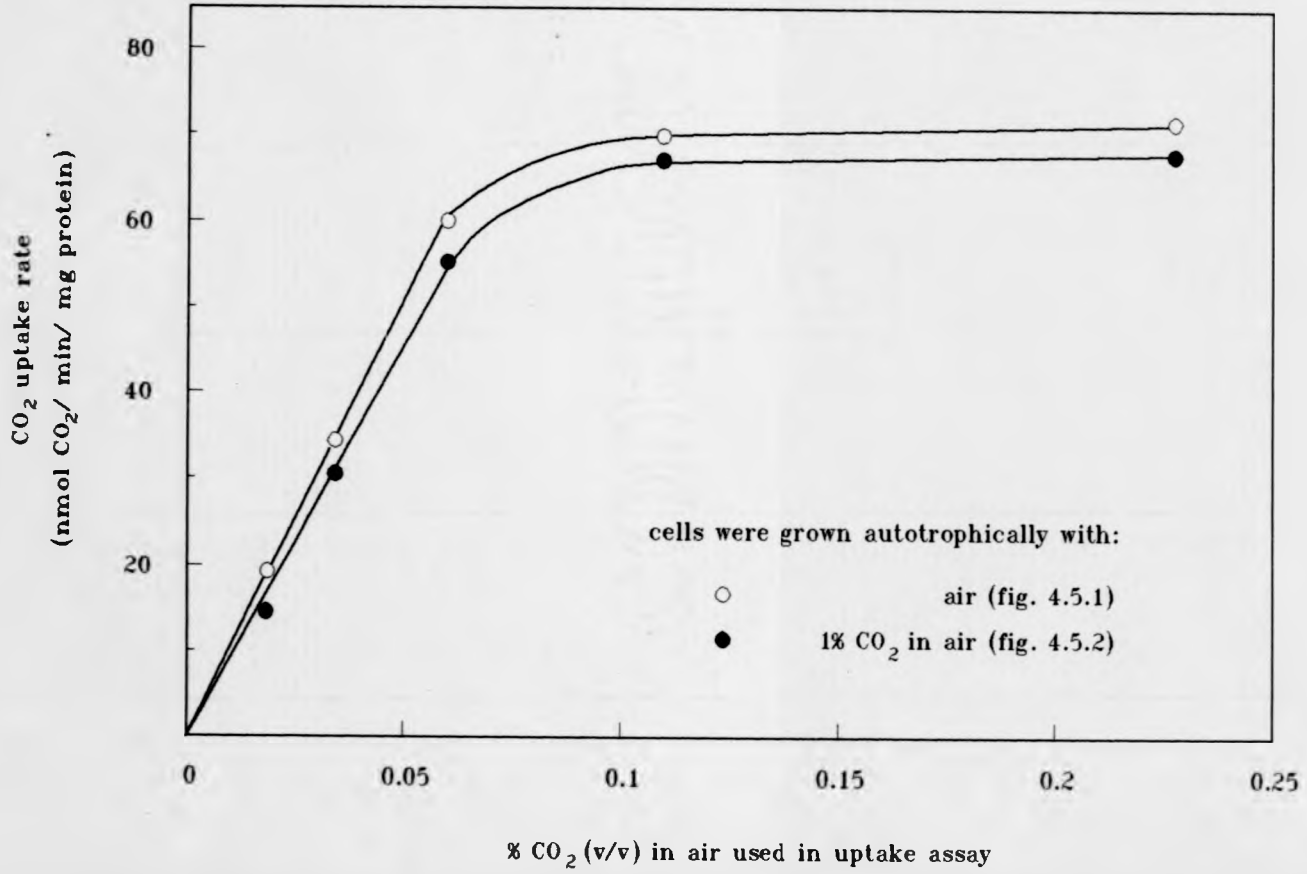
The maximum rate for each CO_2 concentration was converted to nmol CO_2 /min /mg protein and then plotted in figure 4.6.

Figure 4.5.2 Effect of CO_2 concentration on its uptake by strain BC1 grown with 1% (v/v) CO_2 in air (time course experiments). The medium was de-gassed with CO_2-free air and a range of CO_2 concentrations used as in fig. 4.5.1. A time course was then established for each concentration.



The maximum rate for each CO_2 concentration was converted to nmol CO_2 /min /mg protein and then plotted in figure 4.6.

Figure 4.6 Effect of CO_2 concentration on the rate of CO_2 uptake by strain BC1. The maximal uptake rates from fig. 4.5.1 and 4.5.2 were plotted against their respective CO_2 concentrations.



see also Norris, 1989b). It would seem from fig. 4.6 that strain BC1 could achieve a maximum autotrophic iron-oxidation rate when it was capable of transporting 65 to 70 nmol CO_2/ min/ mg protein, but not when the transport capacity was reduced to between 30 and 35 nmol CO_2/ min/ mg protein.

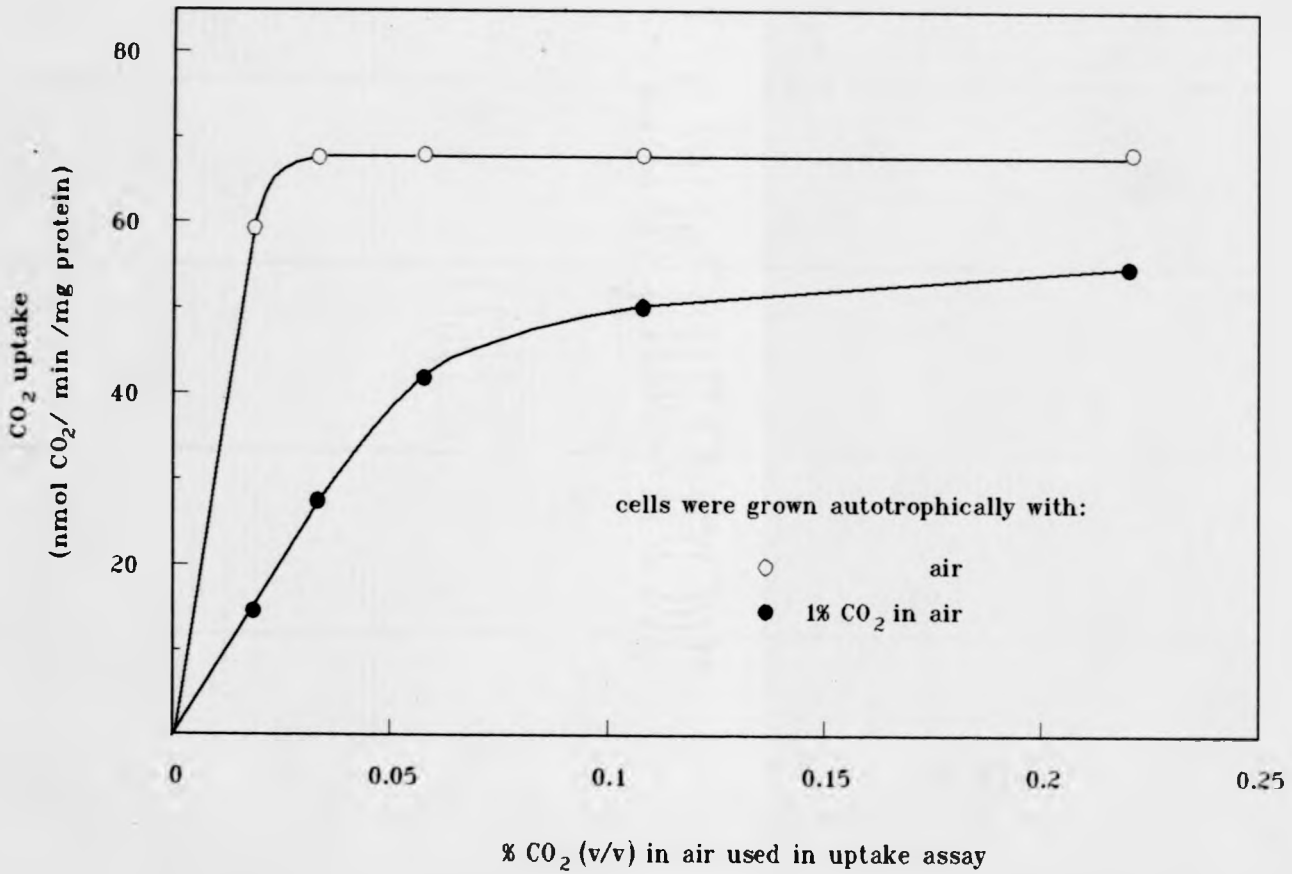
As discussed in section 3.2, it was possible that supplementary carbon may have been supplied by a contaminating carbon source (e.g. from media salts), for growth under air. This possibility was supported by consideration of the very small amount of additional carbon that would be required to support a single population division of strain BC1, "air-grown" biomass (see fig. 3.1).

Wood and Kelly (1985) had previously proposed that the moderate thermophiles were representatives of a physiological type whose metabolism was best described as preferentially mixotrophic, in that growth was less rapid in wholly autotrophic or heterotrophic conditions.

Both autotrophically air-grown and 1% (v/v) CO_2 in air-grown cells of *T. ferrooxidans* (fig. 4.7) were capable of maximum CO_2 transport rates that were similar to those exhibited by strain BC1 (65 to 70 nmol CO_2/ min/ mg protein). However, at an assay concentration of 0.03% (v/v) CO_2, air-grown *T. ferrooxidans* cells were still capable of optimal transport at a rate of 67 nmol CO_2/ min/ mg protein. In contrast, cells that had been grown with 1% (v/v) CO_2 were only capable of transporting CO_2 at a rate of 27 nmol CO_2/ min/ mg protein. This later rate was comparable with that achieved by strain BC1 at the same assay concentration.

Comparison of the transport rates at an assay concentration of 0.03% CO_2 (fig. 4.7) clearly indicated that *T. ferrooxidans* was capable of the induction of a high-affinity CO_2 transport system following an acclimation period to low C_i concentrations.

Figure 4.7 Effect of CO_2 concentration on the rate of CO_2 uptake by *T. ferrooxidans*.



The whole cell $K_m(CO_2)$ values for air-grown and 1% (v/v) CO_2 in air-grown cells of *T. ferrooxidans* (estimated as the concentration of CO_2 required to support half of the maximal transport rate) were 0.01% and 0.04% (v/v) respectively.

The $K_m(CO_2)$ for strain BC1 was 0.03% (v/v). This was identical for both air grown and 1% (v/v) CO_2 grown cells.

The data from fig. 4.6 and 4.7 clearly indicate that the poor CO_2 transport efficiency of strain BC1 from 0.03% (v/v) CO_2 was a significant difference between the two organisms and was likely to be a major factor responsible for the relatively poor autotrophic growth of this organism under air (fig. 3.1 and 3.2).

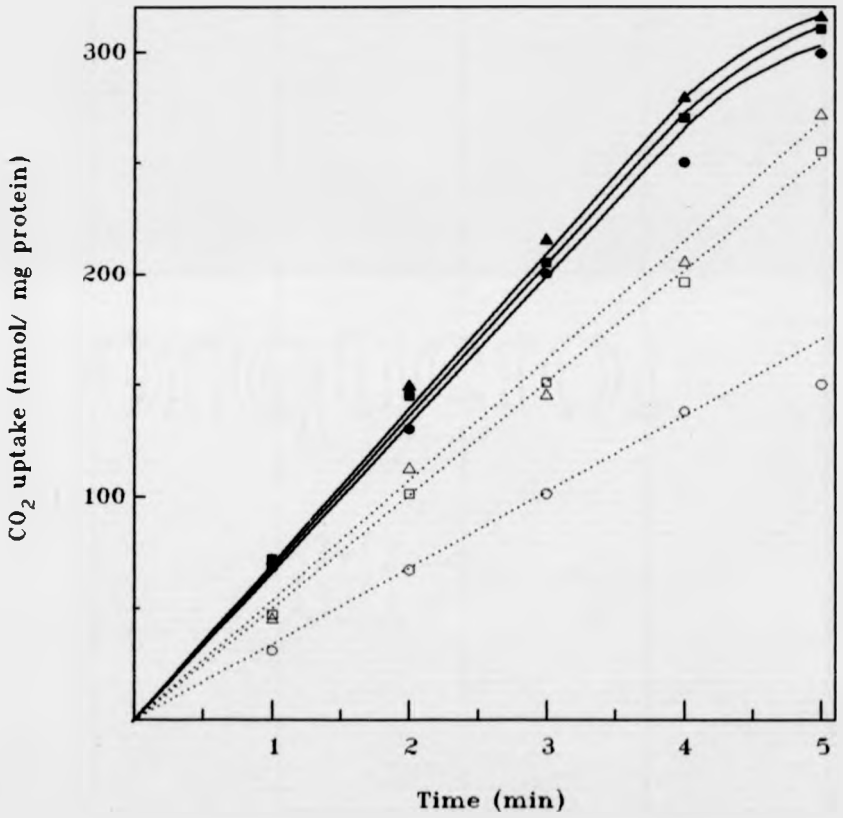
4.6 The C_i pool size in strain BC1 and *T. ferrooxidans.*

Following regeneration, the C_i pool size was assessed in strain BC1 (fig. 4.8).

The total CO_2 transport rates were very similar for all three autotrophically grown cultures, as one would have expected with an assay concentration of 0.1% (v/v) CO_2 (see fig. 4.6). However, the CO_2 fixation rates were consistently lower than their respective total transport rates. Furthermore, the fixation rates appeared to vary inversely with respect to the CO_2 concentration in which the cells had been previously grown. Strain BC1 grown autotrophically with either air or 0.1% (v/v) CO_2 in air demonstrated similar fixation rates of between 40 and 50 nmol CO_2 fixed/ min/ mg protein, whilst those grown with 5% (v/v) CO_2 were only capable of CO_2 fixation at approximately 50% of this rate.

These observed differences were probably a reflection of the amount of RuBisCO, the primary CO_2 fixation enzyme, that was present in strain BC1 during growth at each of the CO_2 concentrations. The permeabilized cell RuBisCO assays for the 5% (v/v) CO_2 and air-grown

Figure 4.8 Strain BC1 C_i pool size investigation. Strain BC1 was grown autotrophically with either air, 0.1%, or 5% (v/v) CO_2 in air. Following harvesting, resuspension and a 4 hour regeneration period, cells from each growth condition were assayed for both total transported carbon and fixed (acid stable) carbon. Labelled bicarbonate was injected to a final concentration of 0.1% (v/v) CO_2.



solid symbols represent total transported carbon
open symbols represent fixed carbon

▲ △ growth with air
■ □ growth with 0.1% (v/v) CO_2 in air
● ○ growth with 5% (v/v) CO_2 in air

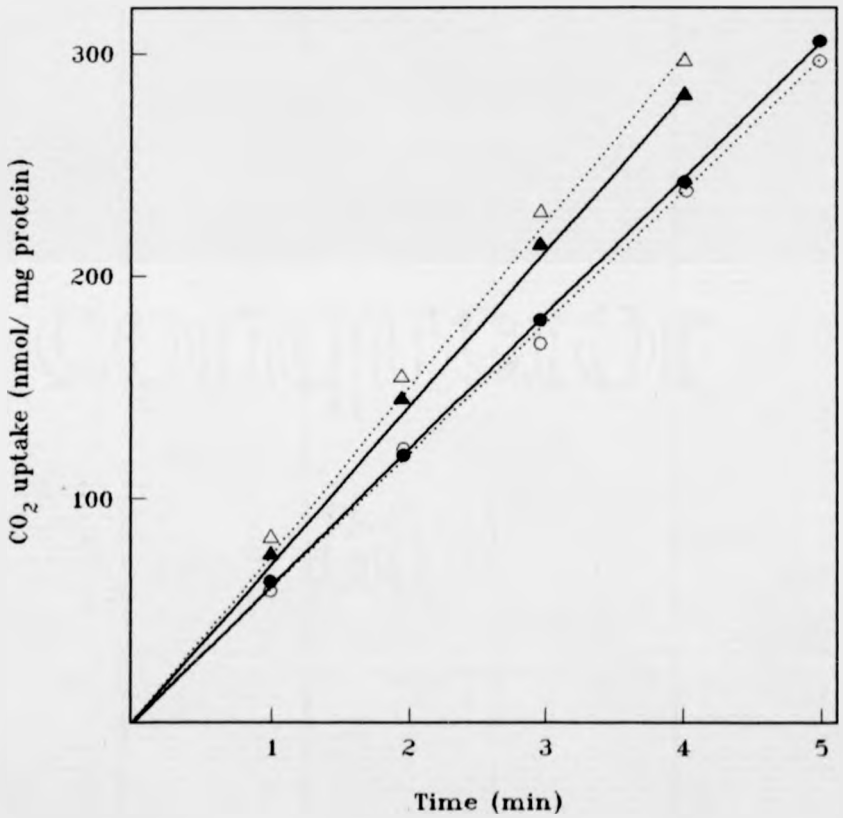
cells were 4.5 and 14.2 nmol CO_2 fixed/ min/ mg protein respectively.

In contrast to strain BC1, the CO_2 fixation and total transport rates were essentially identical in *T. ferrooxidans*, whether the organism had been pre-cultured with either air or 5% (v/v) CO_2 in air.

Clearly, although both organisms were capable of similar total CO_2 transport rates (under excess CO_2 assay conditions), *T. ferrooxidans* was more efficient at fixation of the intracellular C_i pool.

Since both organisms were capable of very similar CO_2 fixation capacities (see chapter 3), this suggested that the availability of CO_2 to RuBisCO was more limited inside strain BC1 than inside *T. ferrooxidans*. This may have resulted from a more rapid interconversion between the intracellular C_i species (CO_2 and HCO_3^-) via carbonic anhydrase in *T. ferrooxidans*. Alternatively, it implicated the efficient intracellular conversion and chanelling of CO_2 to the sites of carboxylation in *T. ferrooxidans*; possibly associated with the functioning of carboxysomes (see fig 1.4) which are known to exist in this organism (see table 1.4).

Figure 4.9 *T. ferrooxidans* C_i pool size investigation. *T. ferrooxidans* was grown autotrophically with either air or 5% (v/v) CO_2 in air. Following harvesting and resuspension, cells from each growth condition were assayed for both total transported carbon and fixed (acid stable) carbon. Labelled bicarbonate was injected to a final assay concentration of 0.1% (v/v) CO_2.



solid symbols represent total transported carbon
open symbols represent fixed carbon

▲ △ growth with air

● ○ growth with 5% (v/v) CO_2 in air

CHAPTER 5

Examination by microscopy for carboxysomes

5.1 Introduction

Carboxysomes have been widely observed in many chemolithotrophic and phototrophic, Calvin cycle prokaryotes. Although the exact function that these polyhedral bodies play is uncertain, their role in C_i fixation has been strongly implicated (see section 1.5 and Price and Badger, 1991).

Several quantitative models for C_i fluxes and photosynthesis have been postulated in cyanobacteria and focus on the carboxysome (whether this be the protein shell or the RuBisCO molecules themselves) as the primary barrier to CO_2 diffusion (Reinhold *et al.*, 1989, Reinhold *et al.*, 1991).

Although there has been considerable controversy as to the cellular location of carbonic anhydrase (CA) activity in cyanobacteria (for e.g. see Lanaras *et al.*, 1985), all of these models make the prediction that such activity is confined to the carboxysomes. The work of Price and Badger (1989a) has provided good evidence for this through the expression of human CA in the cytosol of *Synechococcus*. Such cells lost the ability to accumulate internal C_i as a result of a massive increase in CO_2 efflux and demonstrated a high CO_2-requiring phenotype. Furthermore, the recent work by Price *et al.* (1992) has identified a carboxysome-associated carbonic anhydrase which appears to be the *icfA* gene product (Yu *et al.*, 1992), see section 1.5.

The ultrastructural organization of *T. ferrooxidans* and strain BC1 has therefore been examined, with the presence or absence of carboxysomes the main question to be resolved for the moderate thermophile.

5.2 Examination of strain BC1 and *T. ferrooxidans* by electron microscopy

All cells were essentially prepared according to Kellenberger *et al.* (1958) and sections stained with uranyl acetate.

However, an initial pre-fixation stage with glutaraldehyde (Wood and Kelly, 1989) was used prior to fixation with osmium tetroxide, since this appeared to help reduce cell plasmolysis. A further deviation from the standard protocol was the use of Spurr's resin (see Spurr, 1969). This resin was used in preference to the extensively used araldite because of its associated convenient method of polymerization which simply required the specimen blocks to be baked in an oven at 60°C for 48 hours. In addition, it appeared to confer a greater stability to the sections, especially during high voltage electron microscope examination.

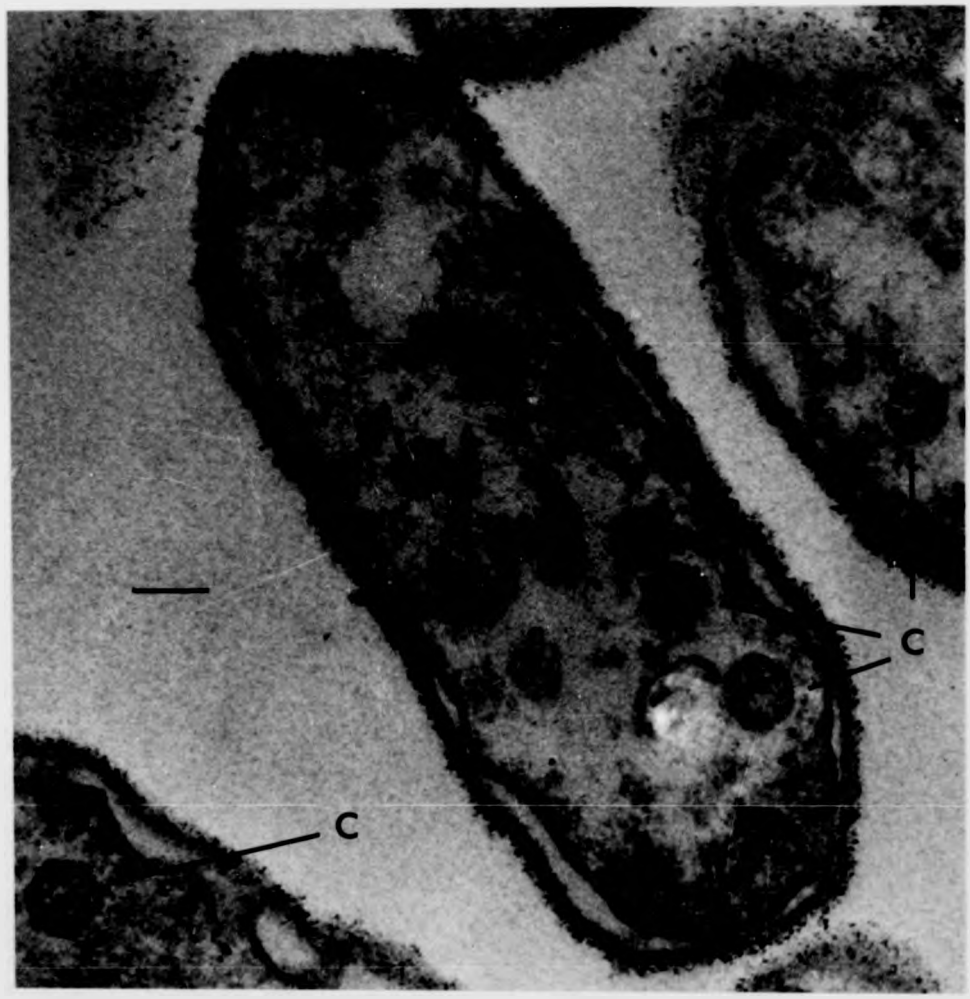
Thin sections of *T. ferrooxidans* (fig. 5.1) illustrated the typical Gram negative membrane present in this organism which was distinguishable from the Gram positive type membrane present in strain BC1 (fig. 5.2b and c). Lutters and Hanert (1989) have previously reported the cell envelope dimensions for *T. ferrooxidans* as; outer and cytoplasmic membranes 6.5 to 7 nm, peptidoglycan 3 to 4 nm thick.

In contrast to earlier reports for this bacterium (Remson and Lundgren, 1966) and other thiobacilli (Shively *et al.*, 1970), there was no evidence for intracytoplasmic membranes.

Autotrophic growth was coupled with the formation of polyhedral bodies. These were of both typical size (100 to 120 nm) and characteristic shape to previous reports for carboxysomes in *T. ferrooxidans* (Lutters and Hanert, 1989) and various other thiobacilli (Lanaras *et al.*, 1991; Holthuijzen *et al.*, 1986; see also Codd, 1988).

Figure 5.1 Thin section electron micrograph of *T. ferrooxidans*.

40 000 x magnification was used to examine thin sections of *T. ferrooxidans* cells grown with 50 mM ferrous iron and gassed with air levels of CO_2.



Bar represents 0.1 μm

C indicates carboxysomes

Although carboxysomes were often difficult to observe, when visible, each cell section typically exhibited between 5 and 8 of these structures. Because of such identification problems it was not possible to ascertain whether their number was increased during growth with limiting CO_2, as has been well documented for other autotrophic bacteria (see section 1.5).

No clear evidence was provided for the presence of storage granules in mid-log phase cells of *T. ferrooxidans*. However, this organism has previously been reported to produce glycogen, but not poly-β-hydroxybutyrate, as a storage material (Lutters and Hanert, 1989).

Thin sections of the moderate thermophile, strain BC1, were examined following autotrophic growth with 50 mM ferrous iron and air levels of CO_2 (fig. 5.2a, b and c) and chemolithoheterotrophic growth with 50 mM ferrous iron and 0.025% (w/v) yeast extract (fig. 5.3a and b).

Comparison of fig. 5.2 (a, b and c) and fig. 5.3 (a and b) illustrates the change in size exhibited by this organism according to its mode of nutrition. Chemolithoheterotrophically-grown cells were notably larger (typically 3.2 to 3.5 µm by 0.8 to 1 µm), whilst cells grown autotrophically varied from 1.9 to 2.5 µm in length and 0.6 to 0.8 µm in width. The size of autotrophically grown cells varied further according to the concentration of CO_2 supplied; those grown with air levels of CO_2 were generally longer and narrower than cells grown with excess CO_2 (results not illustrated). Strain BC1 cells were significantly larger than those of the mesophile, *T. ferrooxidans* (typically 1.3 µm by 0.4 µm, see fig. 5.1).

Of major interest to this work, and in contrast to *T. ferrooxidans*, autotrophic growth of strain BC1 was not coupled with the formation of any cytoplasmic structures resembling carboxysomes (fig. 5.2a, b and c).

Figures 5.2 Thin section electron micrographs of strain BC1 grown autotrophically with 50 mM ferrous iron and gassed with air levels of CO_2.

a) 10 000 x magnification used in microscopy

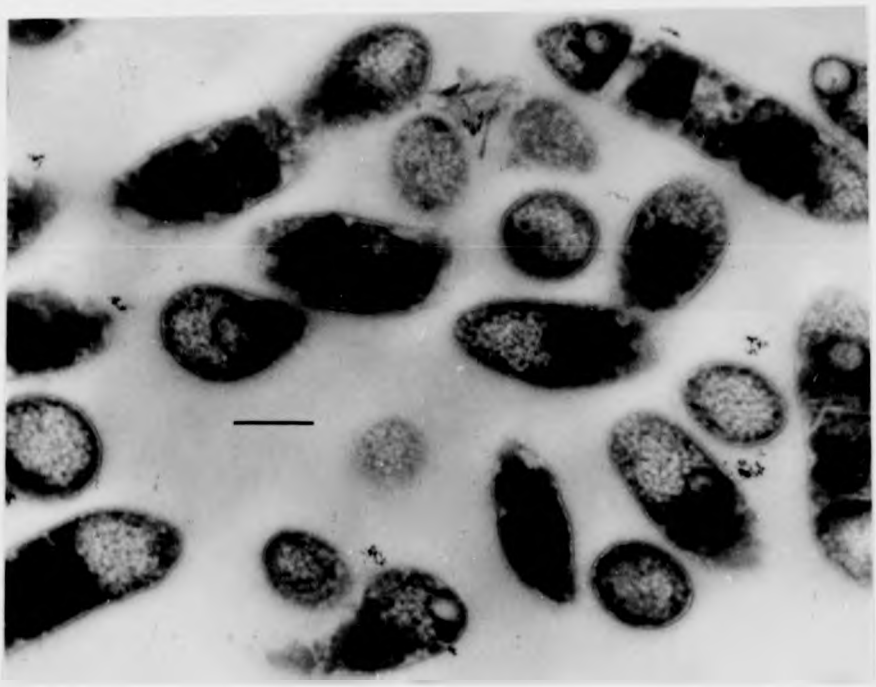
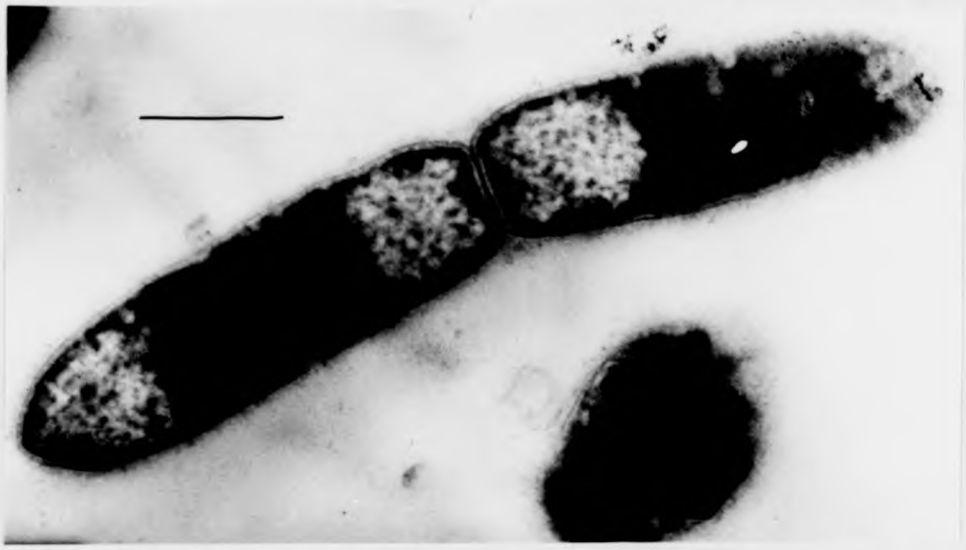
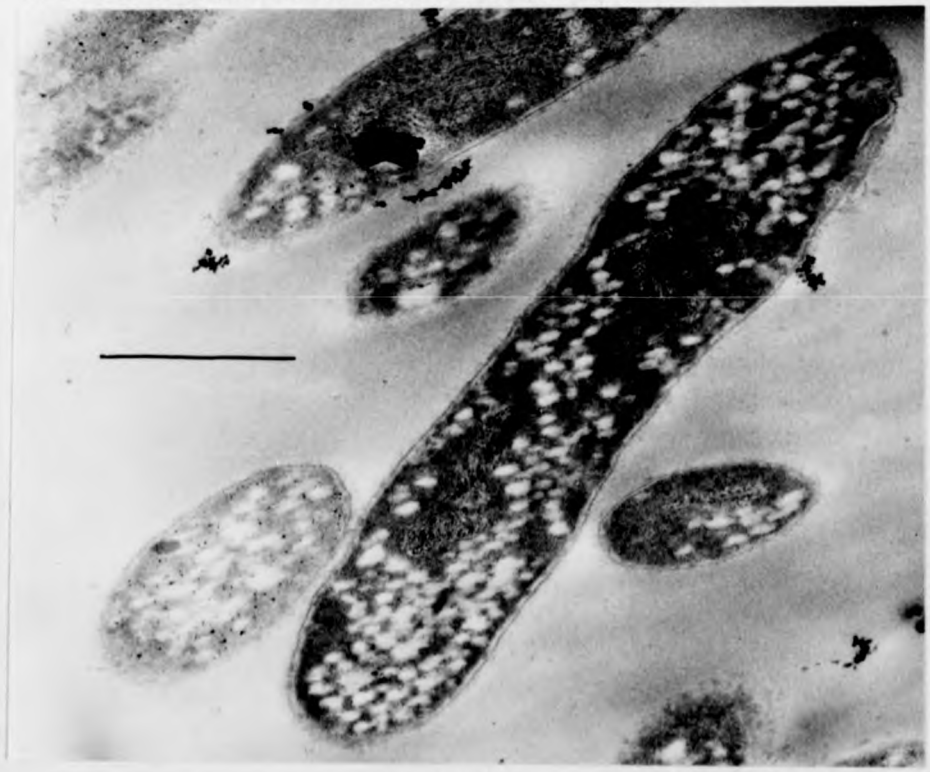


Figure 5.2 (a, b and c) bar represents 0.5 μm

b) 20 000 x magnification used in microscopy

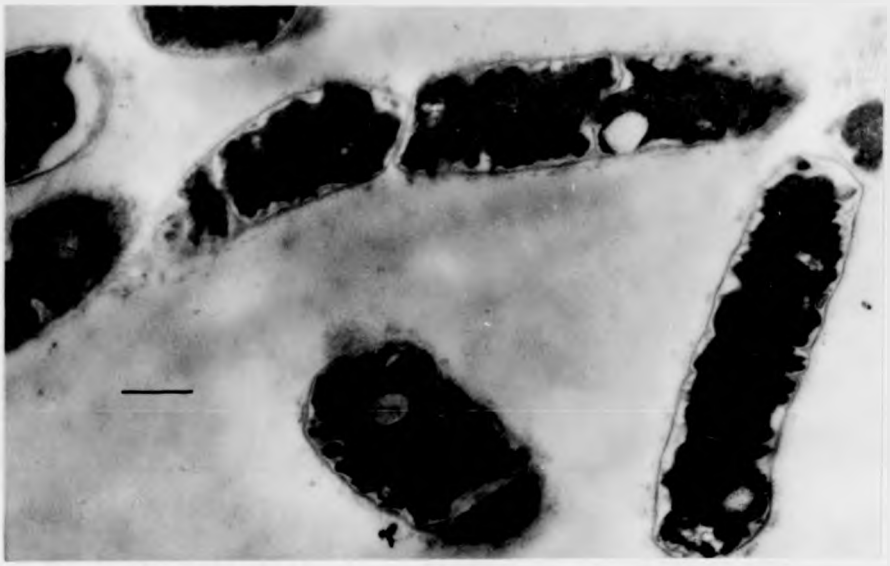


c) 25 000 x magnification used in microscopy

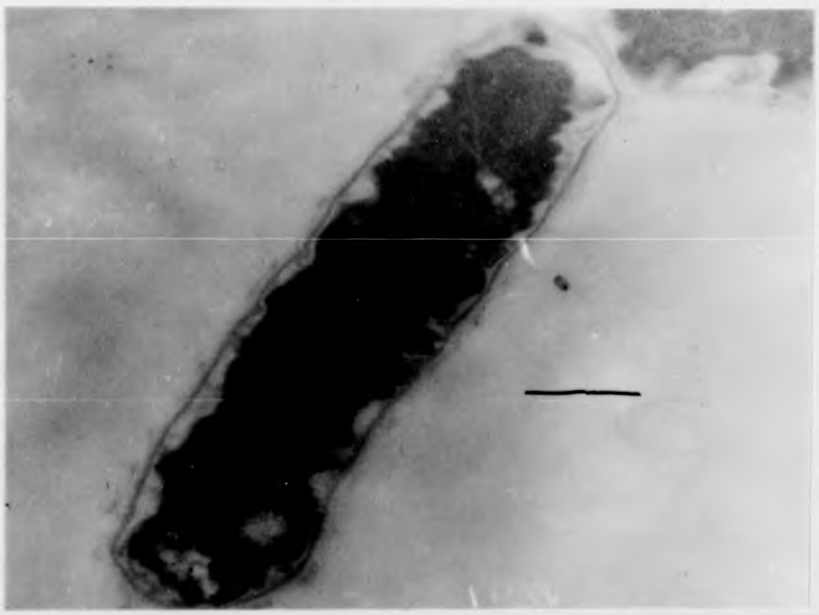


Figures 5.3 Thin section electron micrographs of strain BC1 grown chemolithoheterotrophically with 50 mM ferrous iron and 0.02% (w/v) yeast extract.

a) 10 000 x magnification used in microscopy



b) 20 000 x magnification used in microscopy



Figures 5.3 (a and b) bar represents 0.5 μm

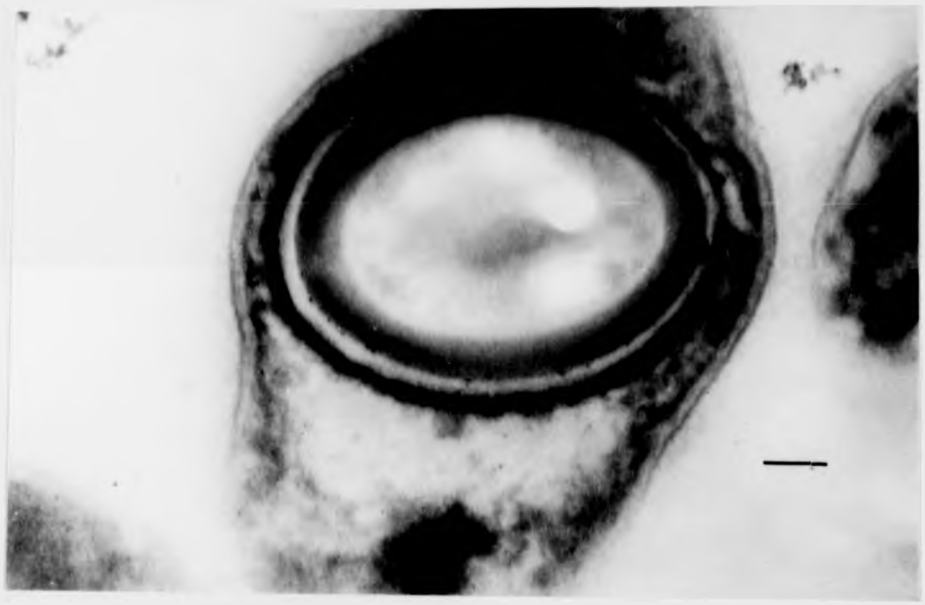
Consistent with the Gram positive nature of strain BC1 was the absence of an external membrane on the cell wall, the latter reaching a thickness of between 20 and 25 nm.

A comparison of fig. 5.2 and 5.3 indicated the accumulation of a negatively staining material during autotrophic growth that was apparently absent during chemolithoheterotrophic growth. This material was almost completely absent in sections of strain BC1 grown with 5% (v/v) CO_2 (results not illustrated), although large quantities were clearly visible, especially at the poles of air-grown cells (fig. 5.2a,b and c). Furthermore, the appearance of this material in response to CO_2 limitation suggested that it may have represented a source of nitrogen (possibly comparable to cyanophycin in cyanobacteria; see Allen, 1984) or phosphorus storage (polyphosphate bodies; see also Allen, 1984), possibly indicating a very early onset of sporogenesis (see fig. 5.4).

In common with *Sulfobacillus thermosulfidooxidans* (Golovacheva, 1979), which is probably the same species as strain BC1 (see section 1.2.2), various cytoplasmic "lamellar-like" structures were occasionally visible in thin sections of strain BC1 (fig. 5.2c). Both organisms were capable of forming spores in which the protoplast was enclosed within a thick cortex containing multilayer coats very similar in structure to *Bacillus* endospores. Such spores in *S. thermosulfidooxidans* have been reported to be resistant to 30 minutes of heating at 110°C (Golovacheva, 1979). Flagellar apparatus was consistently absent from both bacteria, although motility has been observed occasionally with strain BC1. *S. thermosulfidooxidans* has been observed to form a distinctive and unique type of appendage that functions in anchoring this organism onto mineral sulphide substrates (Golevacheva, 1979). No comparable structures, as expected, were seen with ferrous iron-grown strain BC1.

Figure 5.4 Thin section electron micrograph of strain BC1 spore structure.

50 000 x magnification of strain BC1 at a late stage in sporogenesis. Cells had been grown autotrophically with 50 mM ferrous iron and gassed with air levels of CO_2.



Bar represents 0.1 μm

Finally, an abundant slime capsule with a thickness of up to 0.3 μm has been reported in close association with the cell wall of *S. thermosulfidooxidans* (Golevacheva, 1979). However, its apparent absence in strain BC1 (fig. 5.2 and 5.3) was consistent with observations of *S. thermosulfidooxidans* which also lacked this layer when ferrous iron was the sole energy source (Golevacheva, 1979).

CHAPTER 6

Thiobacillus ferrooxidans cell fractionation and putative, low C_{l^-} induced polypeptides.

6.1 Introduction

It remains uncertain whether a common system or separate systems are responsible for transport of the two C_i species, CO_2 and HCO_3^-, in cyanobacteria and microalgae, with evidence existing for both (see Kaplan *et al.*, 1991). Extensive biochemical and molecular analyses through *in vivo* labelling studies and characterization of high C_i-requiring mutants have allowed the identification of several polypeptides that are believed to be involved in the acclimation response to low C_i in these organisms (see Coleman, 1991).

In contrast, there is a scarcity of data available on C_i transport and no information regarding proteins involved in the process in chemolithotrophic bacteria.

Having established the presence of an inducible, high affinity CO_2 transport system in *T. ferrooxidans* (chapter 4), attempts have been made to identify some of its potential polypeptide components.

T. ferrooxidans was grown with 50 mM ferrous iron in 20 L pots and gassed with either air or 5% (v/v) CO_2 in air. Following harvesting, cells were lysed at either pH 2.5 (the putative periplasmic pH, see Ingledew, 1986) or pH 6.5 (the cytoplasmic pH).

Ferrous iron oxidation provides energy for the growth of a number of acidophilic bacteria and has been most extensively studied in *T. ferrooxidans* (see Ingledew, 1982; Yamanaka *et al.*, 1991; Barr *et al.*, 1990; Mansch and Sand, 1992). Although the cell surface iron oxidase remains to be identified in this organism (both rusticyanin and an iron/sulphur cluster have been implicated here, see Yamanaka *et al.*, 1991), several components of the electron transport pathway from ferrous iron to oxygen have been identified and their cellular location determined. Furthermore, the cell membrane and periplasm of *T. ferrooxidans* contain an unusually high specific content of these

electron transport chain components (Ingledew, 1982). Consequently, the use of cytochrome spectra (dithionite-reduced minus oxidized) provided ideal markers for monitoring the purity of the respective membrane and cell-free fractions in this organism, prior to analysis by SDS-PAGE.

6.2 Whole cell cytochrome spectra

The room temperature, dithionite-reduced minus oxidized difference, spectra of *T. ferrooxidans* whole cells were essentially identical (fig. 6.1), irrespective of whether the cells had been cultured with air or 5% (v/v) CO_2 in air, as would be expected.

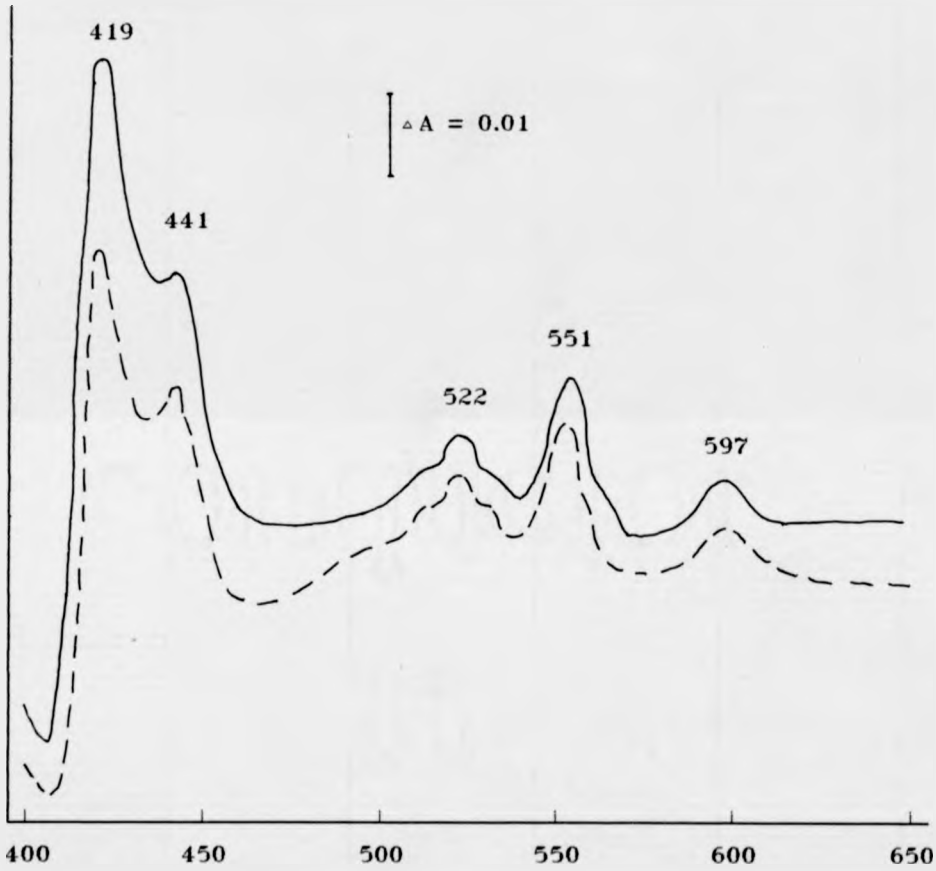
The Soret peak at 441 nm and α-peak absorption at 597 nm could be attributed to one or more a_1-type cytochromes, whilst absorption peaks at 419, 522 and 551 nm corresponded to the Soret, α- and β-bands of *c*-type cytochromes (see Barr *et al.*, 1990).

Low temperature spectra of *T. ferrooxidans* (77 K) have partly resolved the broad absorption band around 551 nm into peaks at 548 and 552 nm with a shoulder at 557 nm; the latter two suggested the presence of cytochromes c_1 and *b* (see Barr *et al.*, 1990).

Comparison of the two spectra (representing air-grown and 5% (v/v) CO_2 grown cells) in fig. 6.2 indicated that slight differences were observed with the relative absorption maxima at 419 nm and 441 nm. Such differences were however commonly observed, and appeared to vary with the amount of added dithionite. These two peaks also exhibited a slight shifting towards 421 nm and 439 nm respectively, with an increase in the assay period (results not illustrated).

Figure 6.1

Room temperature spectra (dithionite-reduced minus oxidized) from whole cell preparations of *T. ferrooxidans* grown with 50 mM ferrous iron and either air or 5% (v/v) CO_2 in air.



solid line represents 5% (v/v) CO_2 grown *T. ferrooxidans*
broken line represents air grown *T. ferrooxidans*

All cells were resuspended in pH 1.6 media.
final concentrations :
0.5 mg protein/ ml for air grown cells
0.6 mg protein/ ml for 5% (v/v) CO_2 grown cells

6.3 Membrane-free, soluble extract cytochrome spectra

The removal of membranes from the soluble fractions (see 2.20) was monitored through the disappearance of the a_1-type cytochromes and their associated absorption maxima at 441 nm and 597 nm (see fig. 6.2.1 and 6.2.2 for 5% (v/v) CO_2-grown and air-grown cells respectively).

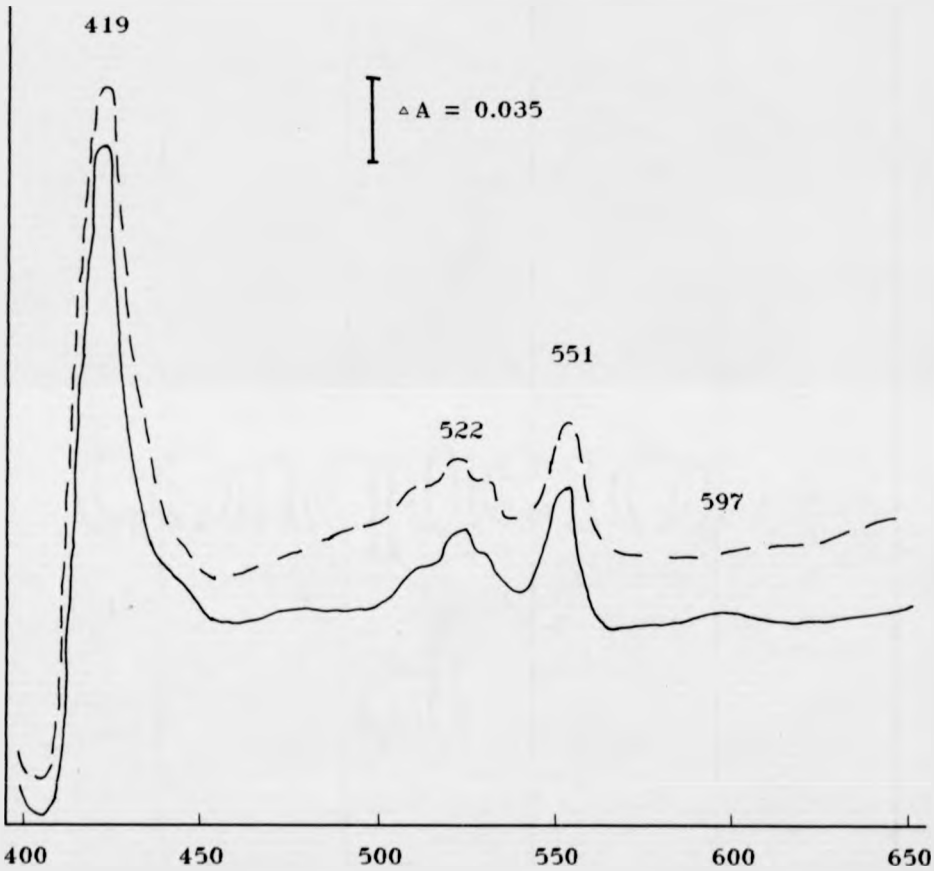
Membrane-free, soluble extracts prepared at pH 6.5 (fig. 6.2.1 and 6.2.2), were devoid of any absorption maxima at 441 nm or 597 nm. This indicated that no detectable membrane contamination had occurred. The three absorption peaks observed at 419, 522 and 551 nm could primarily be attributed to the soluble *c*-552 cytochrome (see Yamanaka *et al.*, 1991).

In contrast to the higher pH preparations that appeared red in colour, membrane-free soluble extracts at pH 2.5 exhibited a pale blue tinge which could probably be attributed to the presence of acid-stable rusticyanin. This is a small, blue copper protein and may account for up to 5% of the total cell protein in *T. ferrooxidans* (see Yamanaka *et al.*, 1991 and Barr *et al.*, 1990).

Both pH 2.5 preparations of membrane-free, soluble extracts derived from either air-grown or 5% (v/v) CO_2 in air-grown cells of *T. ferrooxidans* (see fig. 6.2.1 and 6.2.2) exhibited low, though detectable, absorption peaks at 441 nm and 597 nm. It was possible that these maxima could have been attributed to the rusticyanin signal (see Yamanaka *et al.*, 1991), though it was most likely that they had resulted from a low level of membrane contamination. The low pH would have helped to preserve the a_1-type cytochrome signal in these preparations.

Figure 6.2.1

Room temperature spectra (dithionite-reduced minus oxidized) from membrane-free, soluble extracts of *T. ferrooxidans* grown with 50 mM ferrous iron and 5% (v/v) CO_2 in air. Preparations were made at pH 2.5 and pH 6.5.

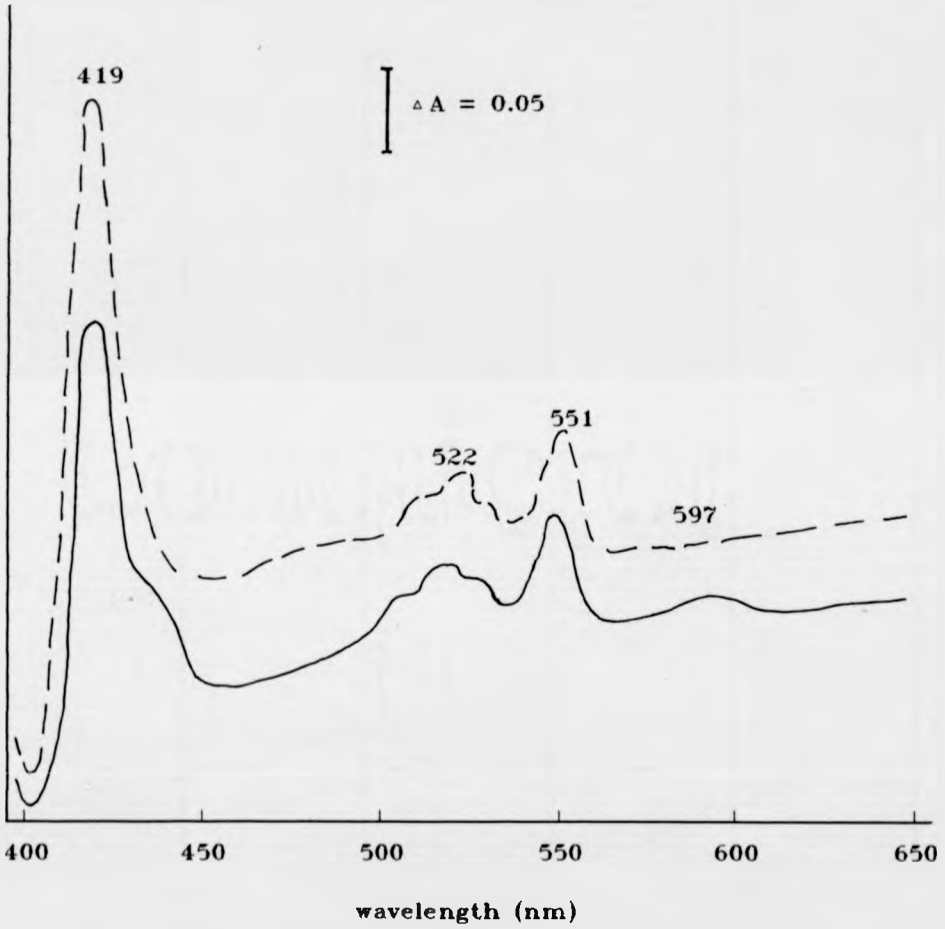


solid line represents preparation A (pH 2.5)
broken line represents preparation B (pH 6.5)

The final protein concentrations were :
0.9 mg/ ml for preparation A
1.5 mg/ ml for preparation B

Figure 6.2.2

Room temperature spectra (dithionite-reduced minus oxidized) from membrane-free, soluble extracts of *T. ferrooxidans* grown with 50 mM ferrous iron and air. Preparations were made at pH 2.5 and pH 6.5.



solid line represents preparation A (pH 2.5)
broken line represents preparation B (pH 6.5)

The final protein concentrations were :
0.9 mg/ ml for preparation A
2.1 mg/ ml for preparation B

6.4 Membrane cytochrome spectra

Washed membranes were resuspended at either pH 2.5 or 6.5 and their dithionite-reduced minus oxidized, cytochrome spectra analysed (see fig. 6.3.1 and 6.3.2 for 5% (v/v) CO_2 in air and air-grown cells respectively).

All membrane preparations exhibited the typical spectra associated with the presence of a_1- and *c*-type cytochromes (see Barr *et al.*, 1990).

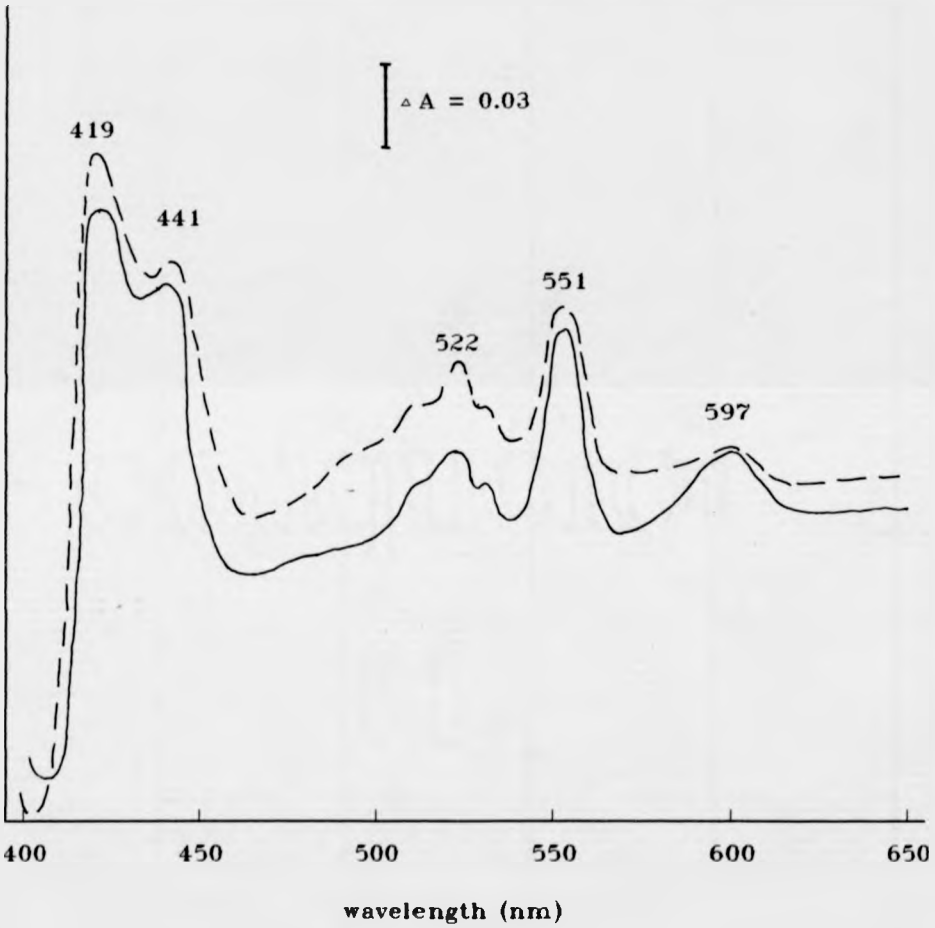
The absorption maxima at 419, 522 and 551 could be attributed to the membrane-bound cytochrome *c*-552 (see Yamanaka *et al.*, 1991), although it was impossible to distinguish this from its soluble counterpart by dithionite-reduced minus oxidized cytochrome spectra alone.

A close examination of the membrane spectra at pH 2.5 and 6.5 (fig. 6.3.1 and 6.3.2), in particular the height of the absorption peak at 597 nm relative to those at 522 nm and 551 nm, suggested that the *c*-type cytochromes were more stable than the a_1-type cytochrome at the higher cytoplasmic pH of 6.5.

An overall comparison of each membrane spectrum with its associated membrane-free, soluble extract counterpart indicated that in all cases good separation of membranes and soluble fractions had been achieved, with little cross-contamination. Each fraction was then further analysed by SDS-PAGE in an attempt to identify low C_i induced polypeptides.

Figure 6.3.1

Room temperature spectra (dithionite-reduced minus oxidized) from soluble extract-free, membranes of *T. ferrooxidans* grown with 50 mM ferrous iron and 5% (v/v) CO_2 in air. Preparations were made at pH 2.5 and pH 6.5.

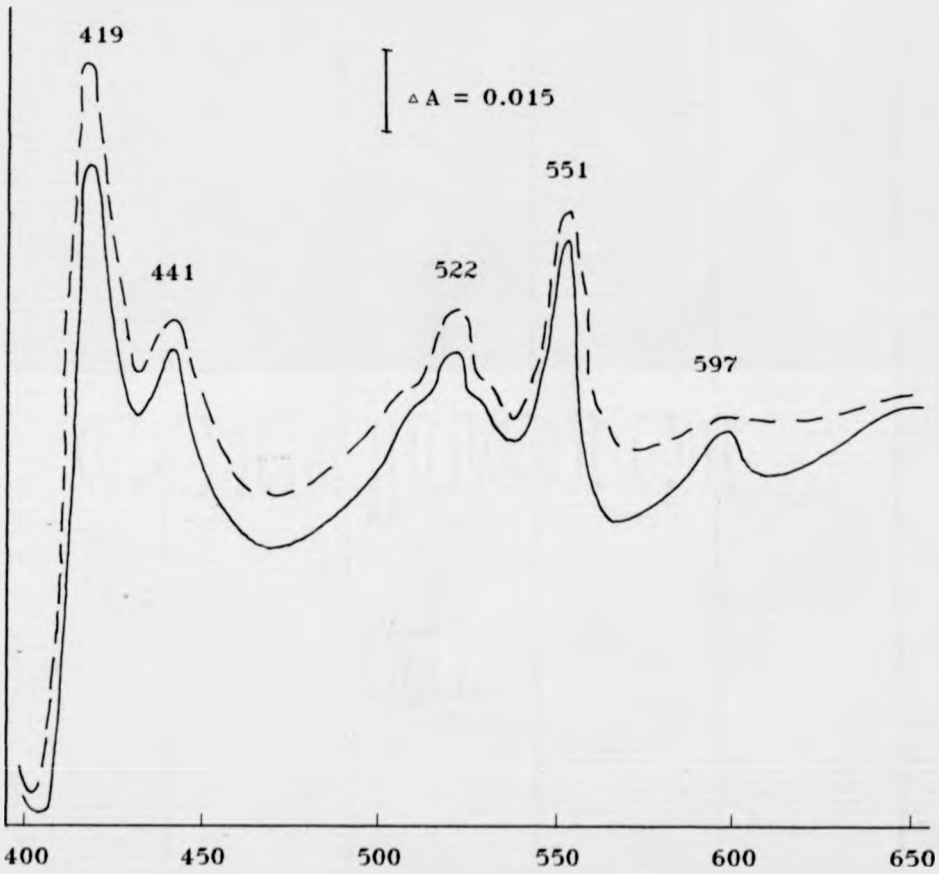


solid line represents preparation A (pH 2.5)
broken line represents preparation B (pH 6.5)

The final protein concentrations were :
0.7 mg/ ml for preparation A
1.0 mg/ ml for preparation B

Figure 6.3.2

Room temperature spectra (dithionite-reduced minus oxidized) from soluble extract-free, membranes of *T. ferrooxidans* grown with 50 mM ferrous iron and air. Preparations were made at pH 2.5 and pH 6.5.



solid line represents preparation A (pH 2.5)
broken line represents preparation B (pH 6.5)

The final protein concentrations were :
0.7 mg/ ml for preparation A
1.1 mg/ ml for preparation B

6.5 SDS-PAGE analysis of membrane and soluble fractions

Membrane-free, soluble extracts from *T. ferrooxidans* were analysed by SDS-PAGE and stained with Coomassie blue (fig. 6.4.1).

In contrast to the pH 6.5 preparations (tracks 6 to 8), lysis at pH 2.5 (tracks 1 to 4) resulted in the denaturation of approximately 50% of the total soluble protein, as apparent from protein determinations prior to electrophoresis. This was removed by centrifugation at 12 000 x g for 15 minutes. The soluble extract had been prepared at pH 2.5 in order to stabilize any periplasmic or loosely associated membrane proteins which might require acid conditions to prevent denaturation.

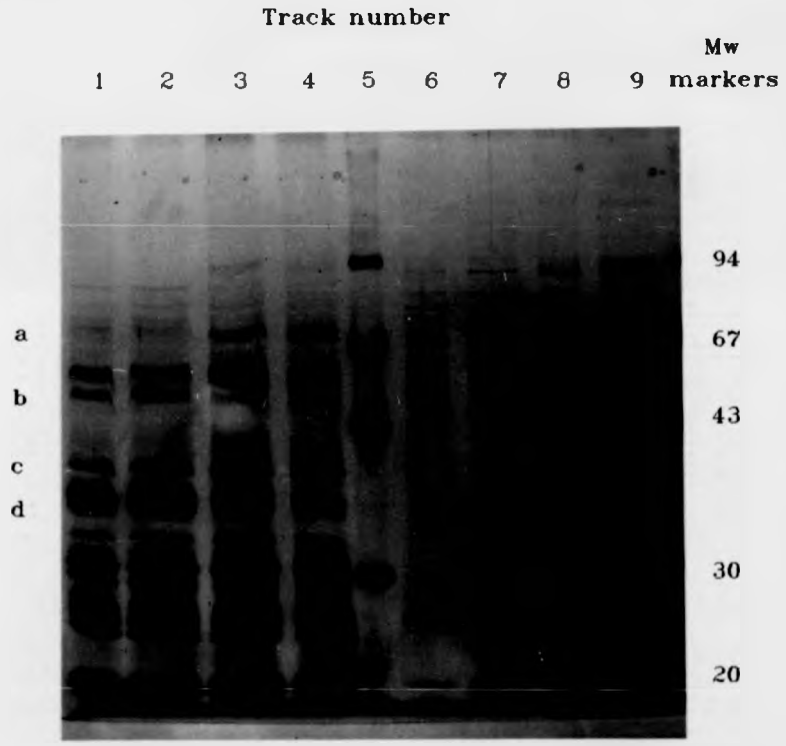
The acid-stable, soluble protein preparations were dominated by polypeptides corresponding to approximate molecular weights of 19, 25, 30, 38-40 and 62 kDa. Polypeptides of similar molecular weight were also prevalent in the membrane preparations (fig. 6.4.2) and have previously been identified as heme-containing proteins (Mansch and Sand, 1992).

Comparison of the acidic, soluble protein preparations (tracks 1 and 2 with tracks 3 and 4) suggested that the quantity of two polypeptides (approximate Mw 70 kDa and 39 kDa; labelled a and c respectively) increased notably following growth of *T. ferrooxidans* with air levels of CO_2. In contrast, the synthesis of a 36 kDa polypeptide (labelled d) appeared to be reduced.

A similar comparison of the high pH preparations (tracks 6 and 7 with those of 8 and 9) indicated that RuBisCO (54 kDa), labelled b, was the only pH 6.5-stable, soluble protein that appeared to be derepressed following low C_i acclimation, see also fig. 3.3.

Figure 6.4.1 SDS-PAGE analysis of membrane-free, soluble extracts from *T. ferrooxidans*.

T. ferrooxidans was grown with 50 mM ferrous iron and gassed with either air levels or 5% (v/v) CO_2 in air. Membrane-free, soluble extracts were then prepared from these cells at pH 6.5 or 2.5 and analysed by 10% (w/v) SDS-PAGE, followed by Coomassie blue staining.



Track number

1 and 2	soluble extract (pH 2.5) from 5% (v/v) CO_2 grown cells
3 and 4	soluble extract (pH 2.5) from air grown cells
5	Molecular weight markers (92, 67, 43, 30 and 20 kDa)
6 and 7	soluble extract (pH 6.5) from 5% (v/v) CO_2 grown cells
8 and 9	soluble extract (pH 6.5) from air grown cells

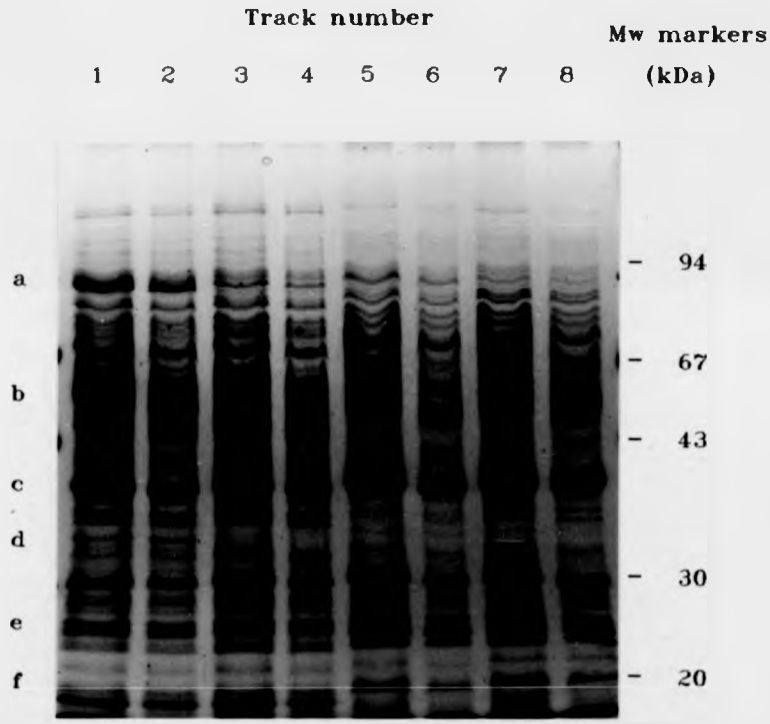
Fig. 6.4.2 illustrates the protein profiles of washed membranes from *T. ferrooxidans*.

In contrast to the soluble protein profiles (fig. 6.4.1), though not surprisingly, the membrane proteins generally appeared to be more stable at pH 2.5 (comparison of tracks 1 to 4 with 5 to 8 of fig. 6.4.2). However, certain polypeptides corresponding to approximate molecular weights of 40, 25 and 21 kDa (labelled c, e and f respectively) appeared to be notable exceptions to this trend. The greater stability of these polypetides at pH 6.5 may have indicated that they were anchored on the cytoplasmic side of the inner membrane.

Following protein correction for loading differences, comparison of tracks 1 and 2 with those of 3 and 4 indicated that three acid stable polypeptides (approximate Mw 93, 62 and 35 kDa; labelled a, b and d respectively) were derepressed after low C_i acclimation. Although the 50 to 70 kD region of fig. 6.4.2 was not very clear, comparison of the higher pH preparations (tracks 5 and 6 with 7 and 8) suggested that similar observations were only apparent for the 93 kD and 35 kDa polypeptides (labelled a and d).

The latter of these two polypeptides was close to the molecular weight of a 36 kDa component identified in *Chlamydomonas reinhardtii* (see Coleman, 1991). Expression of this polypeptide in *Chlamydomonas* was also found to parallel the induction of low C_i acclimation and appeared to be under transcriptional control.

Figure 6.4.2 SDS-PAGE analysis of soluble extract-free, membranes from *T. ferrooxidans*.
T. ferrooxidans was grown with 50 mM ferrous iron and gassed with either air levels or 5% (v/v) CO_2 in air. Purified membranes were then prepared from these cells at pH 6.5 or 2.5 and analysed by 10% (w/v) SDS-PAGE, followed by Coomassie blue staining.



Track number

1 and 2 membrane preparation (pH 2.5) from air grown cells

3 and 4 membrane preparation (pH 2.5) from 5% (v/v) CO_2 grown cells

5 and 6 membrane preparation (pH 6.5) from air grown cells

7 and 8 membrane preparation (pH 6.5) from 5% (v/v) CO_2 grown cells

CHAPTER 7

CO_2 utilization by an autotrophic, thermophilic culture capable of growth with air levels of CO_2.

7.1 Introduction

A moderately thermophilic culture capable of good autotrophic growth on ferrous iron without any enhancement of the CO_2 concentration in air was obtained during the progress of this project (P. Norris, unpublished work).

The culture was a ferrous iron enrichment culture of a sample from an Icelandic hot spring and was shown to contain a variety of bacterial types on examination by light microscopy.

A strain was purified via repeated single colony isolation on solid medium (D. Clarke, unpublished work) containing 20 mM ferrous iron and 0.5 mM tetrathionate.

This strain, designated ICC, was transferred to liquid culture and its autotrophic growth capability then compared with strain BC1 (fig. 7.1).

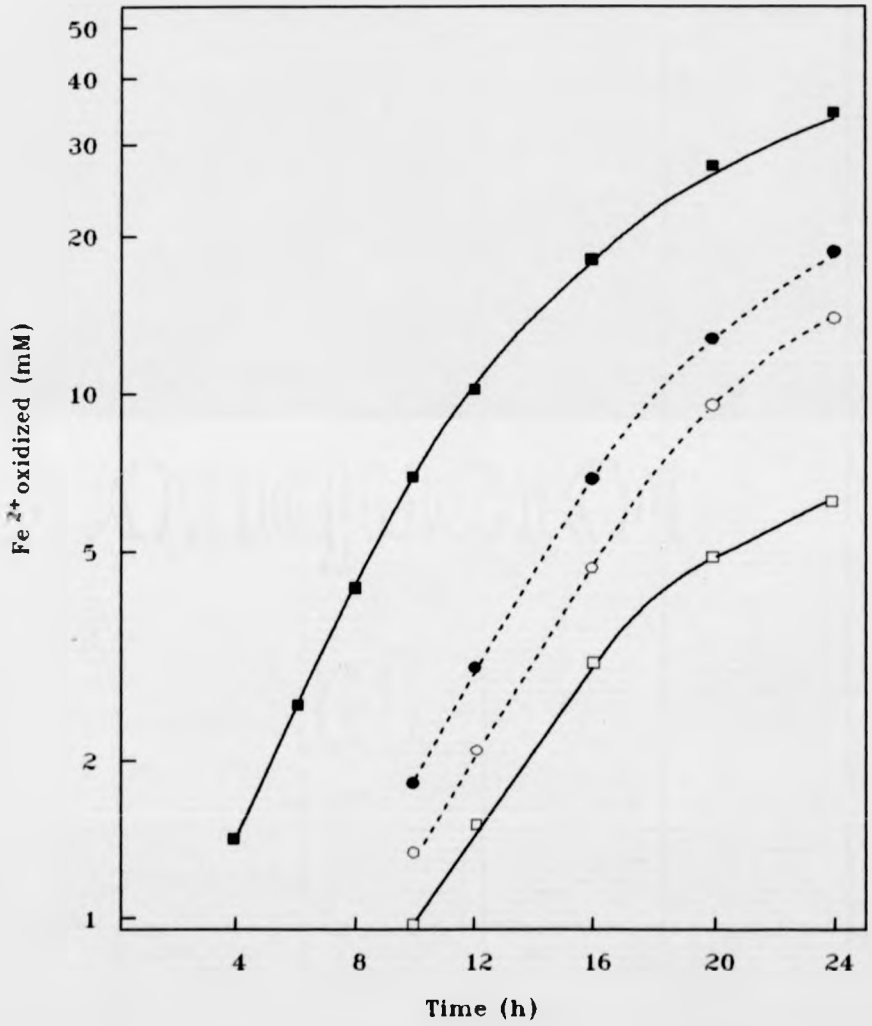
7.2 The effect of CO_2 on the growth of Icelandic culture ICC

Both strain ICC and strain BC1 were grown in 1 litre reactors at 45°C with 50 mM ferrous iron. Gas was supplied as either air or 1% (v/v) CO_2 in air (fig. 7.1). Whilst 1% (v/v) CO_2 in air supported a strain BC1 minimum doubling time of approximately 3 hours, gassing with air alone could only sustain a characteristic short exponential phase of similar rate.

In contrast to strain BC1, though comparable with *T. ferrooxidans* (fig. 3.2), growth of the ICC strain was unaffected when the enhanced CO_2 supply was replaced by air (fig. 7.1). Both autotrophic conditions supported a minimum doubling time of 4 hours for iron oxidation.

In comparison to strain BC1 gassed with 1% (v/v) CO_2 in air a notably longer lag phase was observed with the ICC strain. However, in the absence of supplementary CO_2, strain ICC was clearly capable of considerably more extensive growth than strain BC1 on ferrous iron.

Figure 7.1 Autotrophic growth of strain ICC. Strains BC1 and ICC were grown in 1 litre reactors at 45°C with 50 mM ferrous iron and 0.5 mM tetrathionate. Carbon dioxide was supplied as either air or 1% (v/v) in air, as indicated.



closed symbols represent CO_2 supplied at 1% (v/v) in air
open symbols represent CO_2 supplied as air

strain BC1 ——————
strain ICC ----------

Growth yields of between 250 and 300 mg protein/mole of ferrous iron oxidized were obtained for this culture which were similar to those obtained for both *T. ferrooxidans* and for strain BC1 grown with additional CO_2 (see section 3.2).

In view of the results obtained with *T. ferrooxidans* and strain BC1 which indicated a lack of high affinity CO_2 transport in the thermophile, the important question to address with the ICC culture was whether it was capable of inducing a high affinity C_i concentrating mechanism (CCM) during autotrophic growth with limiting C_i.

7.3 CO_2 transport in strain ICC

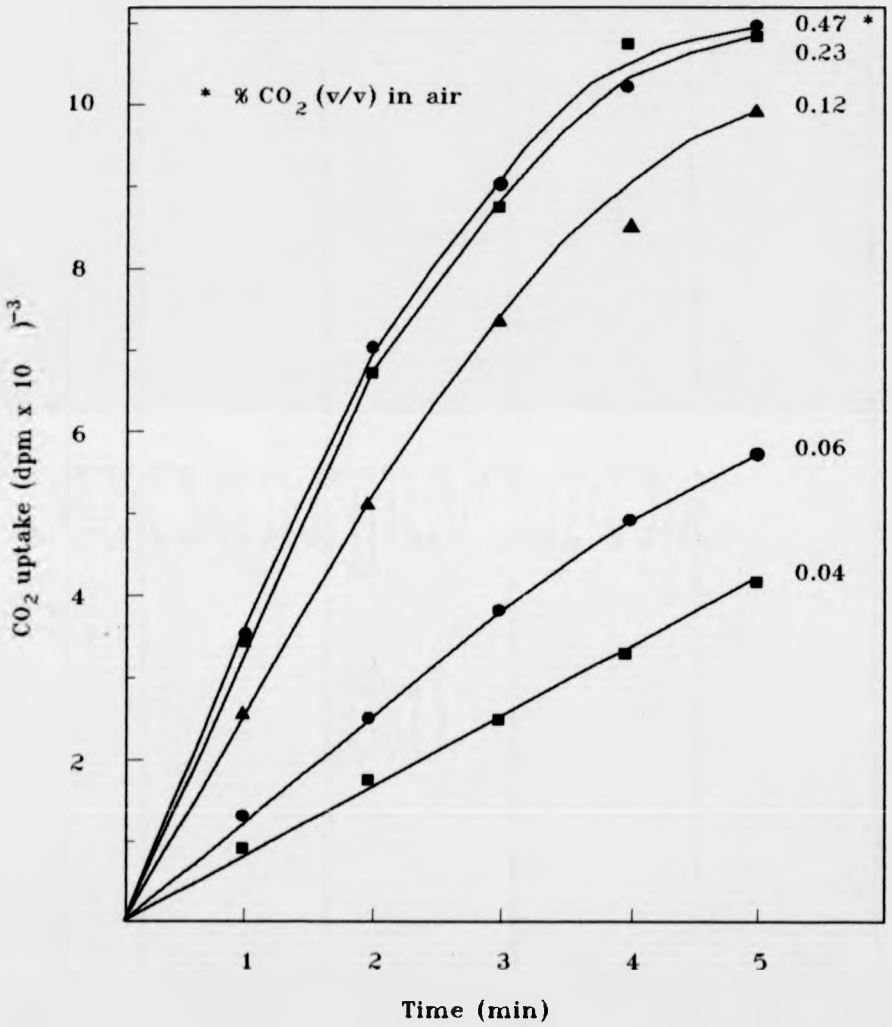
The ICC strain was grown autotrophically in 1 litre reactors (fig. 7.1) and gassed with either air or 1% (v/v) CO_2 in air.

Mid-exponential phase cells were removed and CO_2 transport was assayed over a time course of 5 minutes with a range of CO_2 concentrations (0.04% to 0.47% (v/v) CO_2 in air).

^{14}C-label was rapidly incorporated by the ICC strain (see fig. 7.2.1 for 1% (v/v) CO_2-grown cells; fig. 7.2.2 for air-grown cells) and the maximum rate for each CO_2 assay concentration converted to nmol CO_2/ min/ mg protein. These CO_2 transport rates were then plotted against the respective CO_2 concentrations for each growth condition (fig. 7.3).

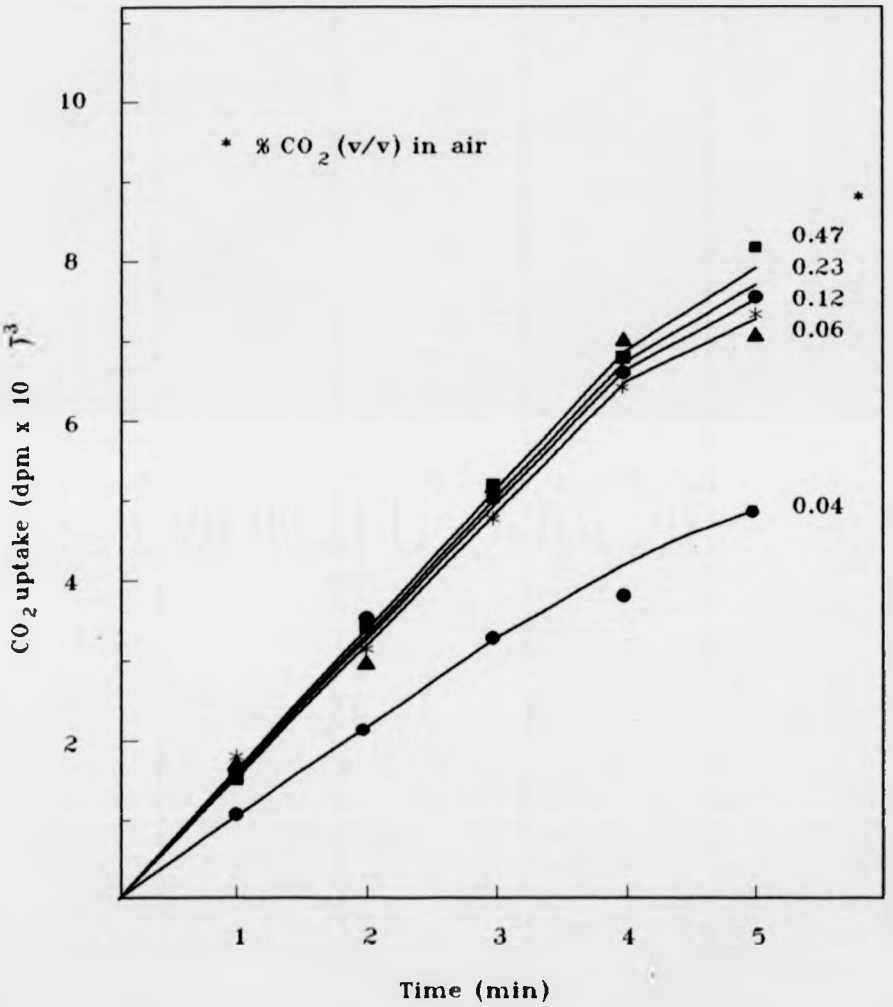
Clear evidence is illustrated for the presence of an inducible CCM in the ICC culture (fig. 7.3), with both air- and 1% (v/v) CO_2-grown cells capable of similar maximal transport rates of between 62 and 65 nmol CO_2/ min/ mg protein. Comparable maximal rates were also achieved by strain BC1 (fig. 4.6) and *T. ferrooxidans* (fig. 4.7).

Figure 7.2.1 CO_2 uptake by strain ICC grown with 1% (v/v) CO_2 in air. Following de-gassing of the medium CO_2-free air, a range of CO_2 concentrations (0.04 to 0.47% (v/v)) were established and time-courses of uptake were determined.



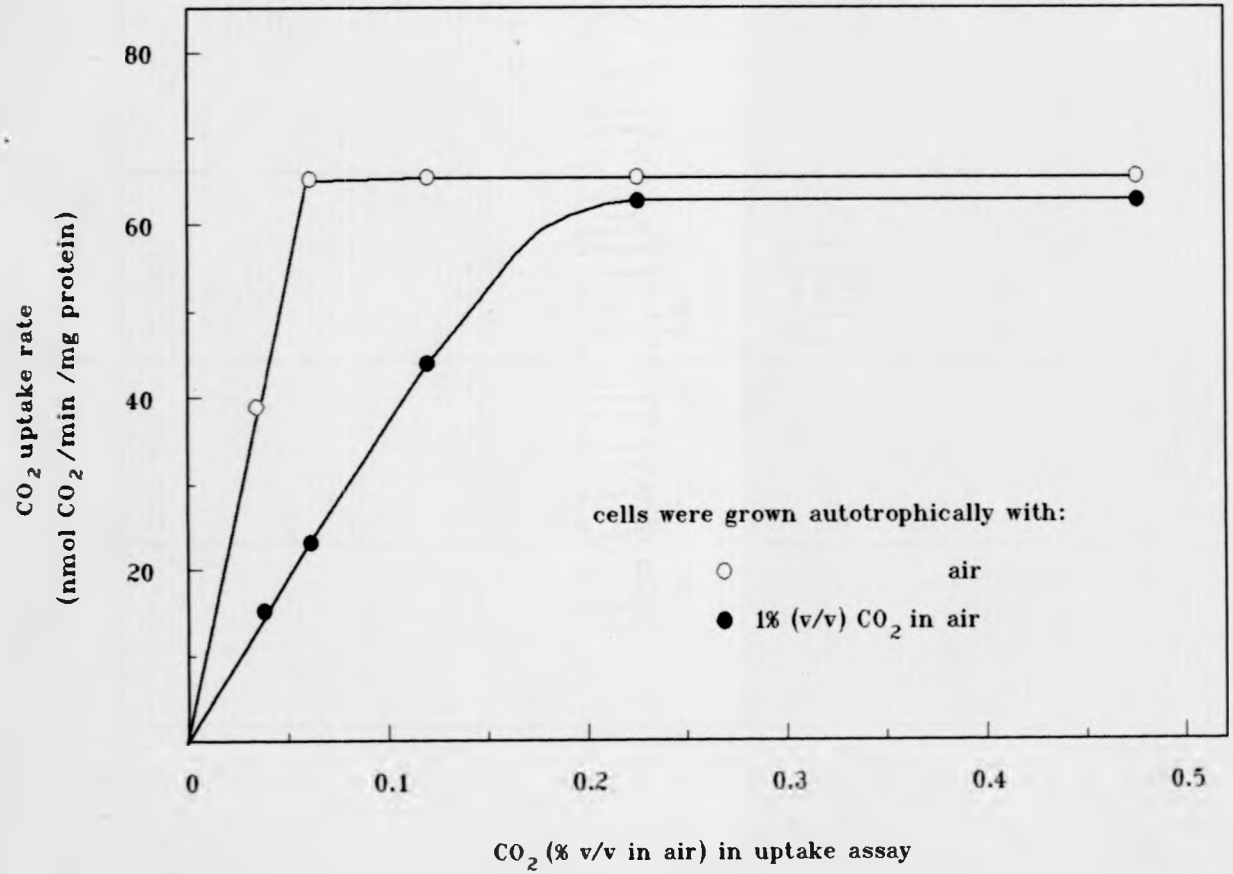
The maximum rate for each CO_2 concentration was converted to nmol CO_2 /min /mg protein and plotted in figure 7.3.

Figure 7.2.2 CO_2 uptake by strain ICC grown with air. Following de-gassing of the medium with CO_2-free air, a range of CO_2 concentrations (0.04 to 0.47 (v/v)) were established. A time-course of uptake was determined for each concentration.



The maximum rate for each CO_2 concentration was converted to nmol CO_2 /min /mg protein and plotted in figure 7.3.

Figure 7.3 The effect of CO_2 concentration on its uptake by strain ICC. The maximum uptake rates generated from fig. 7.2.1 and 7.2.2 were plotted against their respective CO_2 concentrations.



Although all three organisms appeared capable of very similar maximal transport rates, it is possible that a factor had limited potentially greater and perhaps different maximum rates during the assay. Should this have been the case, the most likely candidate would have been the diffusion of CO_2 from the flask void space into the culture medium.

Consideration of a 0.03% (v/v) CO_2 assay concentration indicated that the inducible system present in strain ICC (fig. 7.3) was only capable of transporting 30 nmol CO_2/ min/ mg protein. This corresponded to an approximate 50% saturation of the uptake system and was of similar magnitude to the constitutive and low-affinity system present in strain BC1 (fig. 4.6), although considerably poorer than the inducible, high-affinity system demonstrated by *T. ferrooxidans* (fig. 4.7).

In accordance with the observations of fig. 4.6 and known physiological data relating to strain BC1, it was previously suggested that the capacity for a CO_2 transport rate (under specific assay conditions) of between 30 and 35 nmol CO_2/ min/ mg protein was insufficient to allow good autotrophic growth of this organism with air (see 4.5). However, a similar CO_2 transport rate observed with the ICC culture, which was capable of equivalent iron-oxidation rates to strain BC1 (fig. 7.1) and presumably also fixed CO_2 via the Calvin cycle, was adequate to support the good autotrophic growth of this culture supplied with air (see also section 7.5). This would appear to indicate that the possession of an inducible CCM was the key factor required for optimal CO_2 transport from air levels of CO_2 and that the values obtained for CO_2 uptake rates by experimentation were not necessarily relevant to the *in vivo* (i.e. growth) activity.

The whole cell $K_m(CO_2)$ for the air-grown and 1% (v/v) CO_2-grown ICC culture (estimated as the concentration of CO_2 required to

support half of the maximal transport rate) were 0.03% and 0.08% (v/v) respectively.

7.4 CO_2 transport by resuspended cells of the ICC culture

In view of the obligate "regeneration" requirement of strains BC1 and ALV, following harvesting and resuspension, in order to support CO_2 uptake (fig. 4.2), the moderately thermophilic ICC strain was examined for a similar requirement.

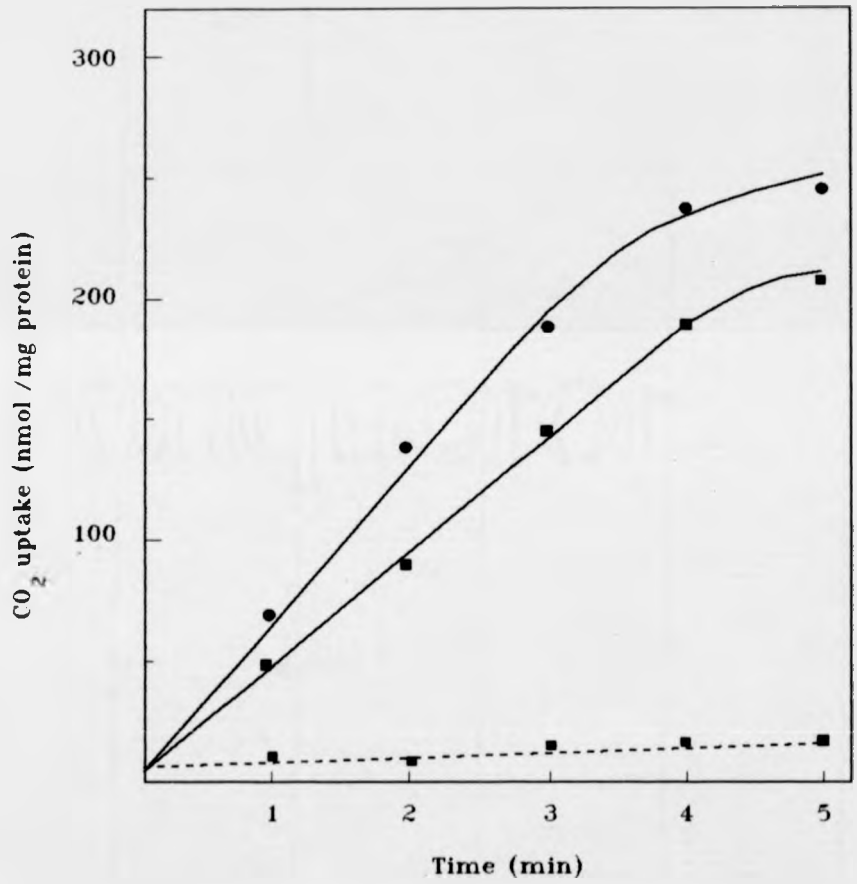
Washed cell suspensions of autotrophically-grown strain BC1 and the ICC culture were incubated at 45°C and their CO_2 transport capacities investigated (fig. 7.4).

^{14}C-label was rapidly incorporated by the ICC strain at rates of 45 and 65 nmol CO_2/ min/ mg protein that were characteristic for 1% CO_2 and air grown cells respectively (fig. 7.3). This clearly demonstrated that "regeneration" was not necessary in order for optimal CO_2 transport to be achieved by this culture.

In contrast, the control incubation of resuspended strain BC1 cells was only capable of transporting CO_2 at the typically poor rate of less than 5 nmol CO_2/ min/ mg protein. However, the full transport capacity of these cells was restored following a four hour "regeneration" period (results not illustrated, but see fig. 4.2).

Whilst both *T. ferrooxidans* (fig. 4.7) and the ICC strain (fig. 7.3) were capable of using induced CO_2 uptake systems, neither required "regeneration" in order to allow maximum CO_2 transport by resuspended cells (fig. 4.1 and 7.4). Conversely, the moderate thermophile strains BC1 and ALV did not possess a high-affinity CO_2 transport system (results only illustrated for strain BC1, fig. 4.6) and both required "regeneration" (fig. 4.2).

Figure 7.4 CO_2 uptake by cell suspensions of strains ICC and BC1. Cultures were grown autotrophically with either air or 1% (v/v) CO_2 in air. The cells were resuspended without a regeneration period and CO_2 uptake measured from an atmosphere of 0.1% (v/v) CO_2 in air in the presence of 50 mM ferrous iron.



7.5 Electron microscope examination of the ICC culture

Further work on the ICC strain, after the preceding uptake experiments had been completed, revealed that the culture was not pure. Thin section transmission electron microscopy (fig. 7.5a and b) clearly indicated that two bacterial types were present in approximately equal numbers.

The first of these bacteria (labelled A, fig. 7.5a) was of similar dimensions (2.5 µm by 1 µm) to autotrophically, air-grown strain BC1 (fig. 5.2) and shared several further morphological features with this organism. It possessed a typical Gram positive cell wall and was capable of forming "*Bacillus*-like" endospores simliar to those observed in *S. thermosulfidooxidans* (Golevacheva, 1979). Organism A also demonstrated the accumulation of a negatively staining compound in response to carbon limitation. As with strain BC1 this appeared to be initially concentrated at the poles, becoming more widespread throughout the cell at the onset of sporogenesis.

Organism B (fig. 7.5b) on the other hand, was considerably narrower (0.3 µm) and clearly distinguishable from the "BC1-like" organism in electron micrographs. No evidence was observed in this second bacterium for either the formation of spores or accumulation of the negatively staining compound, both of which were present in strain BC1 (fig. 5.2) and organism A (fig. 7.5). However, individual heavily stained granules and several other cytoplasmic inclusions were frequently present (fig. 7.5b).

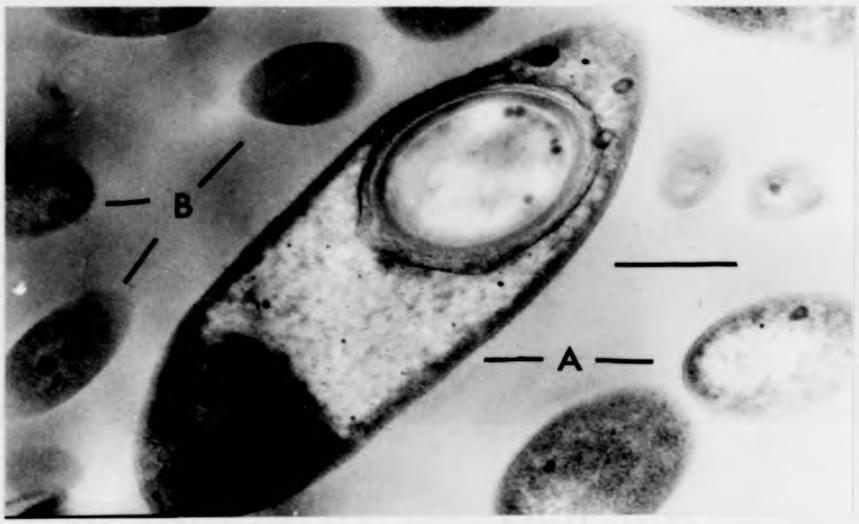
Finally, there was no indication of any internal structures resembling carboxysomes in either of the two bacteria present in the ICC culture.

Discovery that the ICC culture was in fact composed of two distinct bacterial types raised an important question.

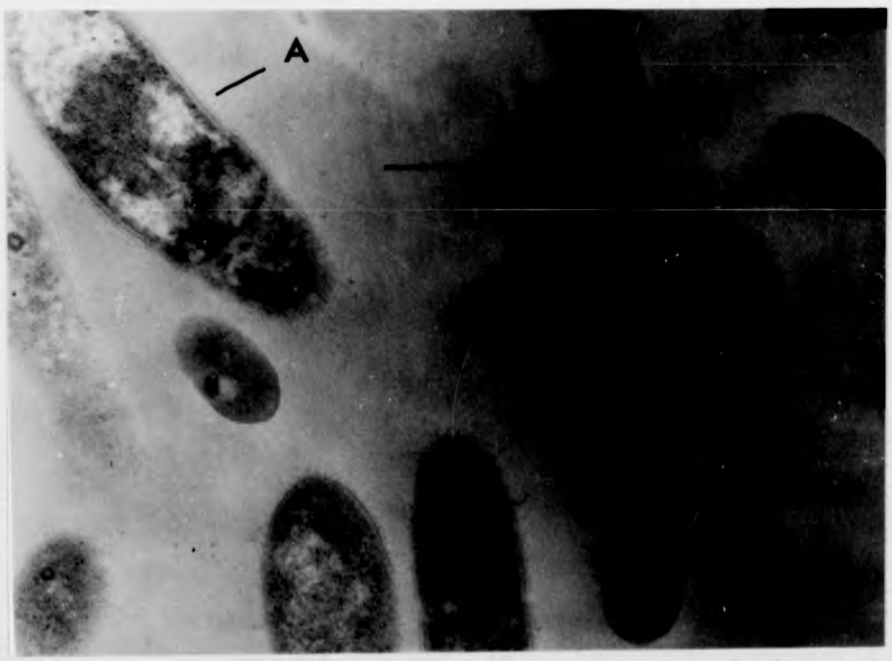
What contribution was each organism making to the high affinity CO_2 transport demonstrated by the ICC strain (fig. 7.3) ?

Figure 7.5 Thin section electron micrographs of strain ICC. Strain ICC was grown autotrophically with 50 mM ferrous iron and gassed with air. Thin sections were stained with uranyl acetate prior to transmission electron microscope analysis. Bar represents 0.5 µm.

a)



b)



It was possible that either one of the two ICC culture bacteria was an organism not contributing to the inducible CO_2 transport system. Consequently, such an organism may have been a heterotrophic or chemolithoheterotrophic scavenger, or an organism with low affinity CO_2 uptake. Consistent with such a model, it is interesting to speculate that had either one of these organisms possessed an inducible, high affinity CCM and constituted 100% of the ICC culture, then at an assay concentration of 0.03% (v/v) CO_2, it may have been capable of CO_2 uptake at a rate of at least 65 nmol/ min/ mg protein; i.e. the CO_2 transport capacity of one of the ICC strain organisms may have been considerably greater than that demonstrated by strain BC1, but its specific uptake capacity could have been hidden by the "contamination" contribution to the biomass. Such a system would then have been comparable in affinity to the *T. ferrooxidans* inducible system (fig. 4.7) and according to previous discussion (see section 4.5 and 7.3), sufficient to support the autotrophic growth of this ICC organism when CO_2 was supplied as air.

When several ICC colonies were picked from the original isolation plate and transferred to liquid medium, all contained the second closely associated organism (D. Clarke, unpublished work). It was therefore possible that the two bacteria may have a commensal partnership in which one of them, perhaps the "BC1-like" organism, excrets a source of fixed carbon that is then available to its heterotrophic partner, organism B.

CHAPTER 8

Summary.

A comparative study of CO_2 utilization by Gram positive, iron-oxidizing, moderate thermophiles and the Gram negative bacterium *Thiobacillus ferrooxidans* was carried out with the aim of explaining the relative incapacity of the thermophiles to grow in the absence of CO_2 concentrations above that in air.

Identification of a feature "preventing" efficient carbon fixation by strain BC1 from air levels of CO_2 was also sought with a view to providing a potential target for later strain improvement in an industrial (mineral sulphide-leaching) context. The study was also intended to reveal further the autotrophic capacity of Gram positive bacteria, a relatively poorly studied phenomenon in such bacteria in comparison with Gram negative organisms.

The results of this study have been summarized in table 8.

No significant differences were observed with the maximal activities of the RuBisCO or PRK enzymes of *T. ferrooxidans* and strain BC1 (table 3.2). In contrast to the situation in *Alcaligenes eutrophus* (see Bowien *et al.*, 1990), following low C_i acclimation, the derepression of RuBisCO alone in strain BC1 and *T. ferrooxidans* (fig. 3.3, table 3.1 and 3.2) indicated that the two Calvin cycle enzymes were independently regulated in both of these organisms.

The very similar RuBisCO substrate affinities (table 3.5) did not account for the capacity of one organism but not the other to demonstrate good autotrophic growth when supplied with air in the absence of CO_2 enrichment.

A combination of the $K_m(CO_2)$ data (table 3.5), 4-30% (w/v) native gel electrophoresis (fig. 3.10) and comparative tube gel assay studies with *Rhodobacter sphaeroides* (fig. 3.19 to 3.21) strongly suggested that both strain BC1 and *T. ferrooxidans* possessed RuBisCO of the form I-type. No evidence was observed for a second RuBisCO form in either

Table 8. Summary of features related to CO_2 utilization by the iron-oxidizing bacteria.

	T. ferrooxidans	Strain BC1	ICC culture
Calvin cycle enzyme activities and affinities sufficient for efficient autotrophic growth	+	+	?
PEP carboxylase activity (permeabilized cells)	+	-	?
High affinity, inducible CO_2 uptake system	+	-	+
Regeneration requirement of harvested cells for CO_2 uptake	-	+	-
Carboxysomes present	+	-	-
Significant unfixed cytoplasmic C_i pool	-	+	?
Good autotrophic growth with CO_2 supplied as air	+	-	+

? not examined

organism.

Initially a ten-fold greater PEP carboxylase was detected in permeabilized *T. ferrooxidans* cells when compared with strain BC1 (table 3.2). Had this observation also been evident in cell-free extracts (table 3.3) then it might have represented a significant difference in the CO_2-fixing potential of the two organisms. However, with the operation of the Calvin cycle, presumably as the primary CO_2 fixation pathway, it is not certain that PEP carboxylase activity would anyway have contributed significantly towards the total CO_2 fixation in either of these bacteria during growth. PEP carboxylase has been shown to play a purely anaplerotic role in both *T. ferrooxidans* (Din *et al.*, 1967) and *T. thiooxidans* (Howden *et al.*, 1972) rather than a significant role in primary CO_2 fixation. The demonstration of upto 20% of the total fixed carbon by this enzyme and its obligate requirement by a *Synechococcus* sp. (Owttrim and Coleman, 1987) so far appears unique in this respect.

Having assessed the major CO_2 fixation enzyme capabilities of strain BC1 and *T. ferrooxidans* and found no appreciable differences, the question of C_i transport was addressed with these bacteria.

Whilst strain BC1 was only capable of CO_2 transport via a constitutive and low-affinity system (fig. 4.6), *T. ferrooxidans* on the other hand, like *T. neapolitanus* (Holthuijzen *et al.*, 1987), possessed an inducible high affinity CO_2 transport system (fig. 4.7). This has not been described previously with *T. ferrooxidans*. The high affinity system allowed *T. ferrooxidans* to continue transporting CO_2 at a maximum rate of between 65 and 70 nmol/ min/ mg protein from an assay concentration of 0.03% (v/v) CO_2 in air (fig. 4.7). In contrast, the poorer affinity system of strain BC1 could only maintain approximately 50% saturation at the same assay concentration.

Clearly this "deficiency" in CO_2 transport by strain BC1 represented a major difference between the two bacteria and was likely to be the one most significant factor responsible for the poor autotrophic growth demonstrated by this organism in culture gassed with air (see fig. 3.1).

This was consistent with the inability of *Thiobacillus versutus*, which also lacked an inducible, high affinity C_i transport system, to grow autotrophically without enhanced levels of CO_2 (Karagouni and Kelly, 1989).

The concurrence of an obligate regeneration requirement by resuspended cells of strain BC1 and strain ALV (fig. 4.2) before CO_2 uptake and the apparent absence of an inducible, high-affinity transport system could be more than coincidence. The results obtained with *T. ferrooxidans* (fig. 4.1 and 4.7) and the ICC strain (fig. 7.3 and 7.4), neither of which required such regeneration but both of which could induce CO_2 transport systems, gave further support to this proposition.

One possibility was that harvesting may have damaged only the low-affinity transport systems of these bacteria. However, following growth of *T. ferrooxidans* under conditions that would only necessitate expression of the low-affinity system (CO_2 supplied in excess), resuspended cells remained capable of optimal transport rates from an assay concentration of 0.1% (v/v) CO_2 in air (see fig. 4.1). This was also true for resuspended strain ICC cells (fig. 7.4), whichever organism in the mixed culture was responsible for the CO_2 uptake. A direct link between the regeneration requirement and absence of an inducible, high-affinity CO_2 transport system in the moderate thermophile strains BC1 and ALV therefore seemed unlikely.

De novo protein synthesis did appear to be essential during regeneration of strains BC1 and ALV. This may have suggested that a

component of their transport system had been physically lost or damaged, and as such was more susceptible to disruption during harvesting than components of the *T. ferrooxidans* or "ICC strain" systems. Alternatively the energy driving component, such as ATP synthesis, may have been inactivated.

An ultrastructural comparison of *T. ferrooxidans* (fig. 5.1) and strain BC1 (fig. 5.2 and 5.3) clearly indicated both the size and cell wall structure differences of these two organisms. Although the precise role that carboxysomes play in C_i fixation remains uncertain (see section 1.5), as suggested by Reinhold *et al.* (1989 and 1991), a selective physiological advantage could be conferred upon *T. ferrooxidans* by the presence of such structures.

In view of the absence of carboxysomes from strain BC1, it was interesting to note the apparent C_i pool-size differences observed in this organism (fig. 4.8) and *T. ferrooxidans* (fig. 4.9).

Despite the two organisms possessing similar CO_2-fixing enzyme capacities when grown with air and also with CO_2 in excess (see table 3.2 and 3.3), the results of fig. 4.8 and 4.9 suggested that *T. ferrooxidans* was more efficient than strain BC1 at utilizing the C_i pool.

This may have resulted from the localized generation of CO_2 within the carboxysomes of *T. ferrooxidans*, as opposed to the general and less efficient cytoplasmic interconversion of C_i species that would have occurred in the absence of these structures in strain BC1. However, this possibility should be viewed with caution because according to the model of Reinhold *et al.* (1991) carbonic anhydrase activity must be located solely within the carboxysomes. Consequently, at least a small, and certainly detectable, pool of un-fixed C_i due to HCO_3^- might have been expected in the cytoplasm of *T. ferrooxidans*.

The most likely explanation for this was that the method employed for determining acid-stable counts had not been as effective at terminating CO_2 fixation in *T. ferrooxidans* as it had been with strain BC1.

In an attempt to identify components involved in the low C_i-inducible, high-affinity CO_2 transport system of *T. ferrooxidans*, the derepression of several polypeptides was observed by SDS-PAGE analysis. The quantity of three membrane polypetides corresponding to approximate molecular weights of 93, 62 and 35 kDa (see fig. 6.4.2) appeared to increase significantly following growth of *T. ferrooxidans* with CO_2 supplied as air. The latter of these was of similar size to a low C_i-induced polypeptide observed in *Chlamydomonas reinhardtii* (see Coleman, 1991).

Two soluble, acid-stable polypeptides (approximately 70 kDa and 39 kDa), probably either periplasmic or loosely membrane associated in origin, also appeared to be increased in quantity following low C_i acclimation (see fig. 6.4.1). This aspect of the study was only of a preliminary nature and not taken further because of its inevitable concentration on *T. ferrooxidans* rather than strain BC1. However, such work could most interestingly be developed with the efficient CO_2-utilizing strain from the ICC culture when this is resolved. Ultimately the use of probes, when sequences of key proteins are available, may be used to identify the genes involved in the inducible CCM of this organism.

A direct comparison of the Icelandic hot spring isolate, ICC, with strain BC1 (fig. 7.1) indicated that the former was capable of both more rapid and a higher final percentage of autotrophic ferrous iron oxidation when air was the source of CO_2. A removal of the necessity for an enhanced atmosphere of CO_2 clearly could give this strain a

higher priority over strain BC1 with respect to commercial application. This capacity of the ICC culture, which was an unexpected find bearing in mind previous unsuccessful searches for such a culture (P. Norris, pers. comm.), obviously reduced the importance (present for most of the period of this thesis work) of having to consider the possibility of manipulating some strain BC1-type improvement with regard to CO_2 utilization.

Electron microscopy of the ICC culture (fig. 7.5) indicated the presence of two bacterial types, one of which shared several morphological features with strain BC1 (fig. 5.2 and 5.3) and the virtually identical *S. thermosulfidooxidans* (Golovacheva, 1979). Clearly, purification of organisms from the mixed ICC culture would allow a detailed comparison of the strains. Furthermore, it would allow determination of each organism's contribution towards the inducible CO_2 transport system demonstrated by the ICC culture (fig. 7.3).

Bearing in mind the absence of an inducible, high-affinity CO_2 transport system in both moderate thermophile strains BC1 and ALV, it would be of great interest if the "strain BC1-like" organism in the ICC culture possessed such a system. Then, the type of experiments which were considered with *T. ferrooxidans*, the search for transport-related inducible polypeptides in the presence of limiting CO_2, would become of prime importance in the investigation of the system in Gram positive bacteria.

However, should the "strain BC1-like" organism in the ICC culture have a CO_2 transport capacity as limited as that of strain BC1, the second organism in the mixed culture would require further characterization. Two possibilities could have to be considered.

Firstly, the organism has a high affinity system for CO_2 uptake which might well resemble that in *T. ferrooxidans* if the uncharacterized thermophile proves to be Gram negative.

Secondly, the uncharacterized thermophile might grow in close association with the "strain BC1-like" bacteria and the iron oxidation capacity of the latter could still be important in the overall characteristics of the mixed culture. Further speculation over these possibilities can await purification of the strains from the mixed culture, although at the time of completing this study and thesis, isolation of pure strains was proving time-consuming and difficult and had currently not been achieved (D. Clarke, pers. comm.).

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