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PATHWAYS OF INORGANIC NITROGEN ASSIMILATION IN CHEMOAUTOTROPHIC BACTERIA–MARINE INVERTEBRATE SYMBIOSES: EXPRESSION OF HOST AND SYMBIONT GLUTAMINE SYNTHETASE

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Summary

Symbioses between chemoautotrophic bacteria and marine invertebrates living at deep-sea hydrothermal vents and other sulfide-rich environments function autotrophically by oxidizing hydrogen sulfide as an energy source and fixing carbon dioxide into organic compounds. For chemoautotrophy to support growth, these symbioses must be capable of inorganic nitrogen assimilation, a process that is not well understood in these or other aquatic symbioses. Pathways of inorganic nitrogen assimilation were investigated in several of these symbioses: the vent tubeworms *Riftia pachyptila* and *Tevnia jerichonana*, the vent bivalves *Calyptogena magnifica* and *Bathymodiolus thermophilus*, and the coastal bivalve *Solemya velum*. Nitrate reductase activity was detected in *R. pachyptila*, *T. jerichonana* and *B. thermophilus*, but not in *C. magnifica* and *S. velum*. This is evidence for nitrate utilization, either assimilation or respiration, by some vent species and is consistent with the high levels of nitrate availability at vents. The ammonia assimilation enzymes glutamine synthetase (GS) and glutamate dehydrogenase (GDH) were detected in all symbioses tested, indicating that ammonia resulting from nitrate reduction or from environmental uptake can be incorporated into amino acids. A complicating factor is that GS and GDH are potentially of both host and symbiont origin, making it unclear which partner is involved in assimilation. GS, which is considered to be the primary ammonia-assimilating enzyme of autotrophs, was investigated further. Using a combination of molecular and biochemical approaches, host and symbiont GS were distinguished in the intact association. On the basis of Southern hybridizations, immunoreactivity, subunit size and thermal stability, symbiont GS was found to be a prokaryote GS. Host GS was distinct from prokaryote GS. The activities of host and symbiont GS were separated by anion-exchange chromatography and quantified. Virtually all activity in symbiont-containing tissue was due to symbiont GS in *R. pachyptila*, *C. magnifica* and *B. thermophilus*. In contrast, no symbiont GS activity was detected in the gill of *S. velum*, the predominant activity in this species appearing to be host GS. These findings suggest that ammonia is primarily assimilated by the symbionts in vent symbioses, whereas in *S. velum* ammonia is first assimilated by the host. The relationship between varying patterns of GS expression and host–symbiont nutritional exchange is discussed.

Key words: chemoautotrophy, symbiosis, hydrothermal vent, nitrogen assimilation, glutamine synthetase, nitrate reductase, glutamate dehydrogenase, amino acid metabolism.

Introduction

Chemoautotrophic bacteria–marine invertebrate symbioses at deep-sea hydrothermal vents, cold seeps and in other reducing environments derive all or part of their nutrition from symbiont chemoautotrophy (Fisher, 1990; Cavanaugh, 1994). To support growth based on sulfide oxidation and carbon dioxide fixation, inorganic nitrogen (e.g. nitrate and ammonia) must also be assimilated. How this occurs has not been well studied and is potentially complicated by the possibility that both host and symbiont are involved in the assimilation of nitrogen.

Chemoautotrophic symbioses have a high demand for nitrogen because of their high biomass (Nix et al., 1995; Lutz and Kennish, 1993) and growth rates (Lutz et al., 1994), and this is matched by the high level of availability of environmental nitrate and, in some cases, ammonia (Johnson et al., 1988; Lilley et al., 1993; Conway et al., 1992). Ammonia and nitrate are likely to be important sources of nitrogen for these symbioses. This is supported by host anatomy, *in situ* water chemistry measurements and physiological studies. In contrast to conventional invertebrate feeding modes, while particulate sources of nitrogen can be utilized by the vent mussel *Bathymodiolus thermophilus* because it is a competent suspension-feeder (Page et al., 1991), these sources are less important to the vent clam *Calyptogena magnifica*, which has a reduced apparatus for feeding (Boss and Turner, 1980; Fiala-Médioni and Métivier,

1986), and are not utilized by *R. pachyptila*, since these tubeworms lack a mouth and gut (Jones, 1985). Dissolved organic nitrogen is not likely to be a significant source since its availability appears to be low. Although few measurements of dissolved organic nitrogen levels have been made at vents (Karl, 1995), amino acid concentrations are generally less than $0.2\,\mathrm{nmol\,l^{-1}}$ around vent communities (Johnson et al., 1988) and less than $0.1\,\mathrm{nmol\,l^{-1}}$ in high-temperature fluids (Haberstroh and Karl, 1989). With the exception of feeding on particles by *B. thermophilus*, inorganic nitrogen is probably the major source of nitrogen to vent symbioses. Similarly, shallow-living symbiotic clams of the genus *Solemya* have reduced or absent feeding structures and digestive system (Reid and Bernard, 1980), and feeding on phytoplankton has been shown to make a minor ($\leqslant 3\,\%$) contribution to the daily nutritional requirements of *S. velum* (Krueger et al., 1992). Given the apparent dependence of the hosts on autotrophy to meet their nutritional requirements, inorganic nitrogen assimilation is a critically important capability.

Assimilation of inorganic nitrogen sources has been demonstrated in a few of these symbioses. In ^{15}N tracer experiments, *R. pachyptila* assimilate nitrate whereas *Solemya reidi* assimilate ammonia and, to a lesser degree, nitrate (Lee and Childress, 1994). At hydrothermal vents, comparisons of *in situ* nitrate concentrations with those of a conservative tracer (silicate) indicate that nitrate is consumed by vent communities (Johnson et al., 1988).

The mechanisms by which these symbioses assimilate ammonia and nitrate are not well characterized, but many of the transformations are probably localized to the bacterial symbionts. Many species of bacteria can assimilate nitrate and ammonia into amino acids (Payne, 1973; Reitzer and Magasanik, 1987). In some species, nitrate serves both as an alternative electron acceptor and as a source of nitrogen (Payne, 1973; Stewart, 1994). In both cases, nitrate is first reduced by nitrate reductase (NR) to nitrite (Stewart, 1994), which can subsequently be reduced to ammonia (Cole, 1988). Bacterial nitrate reductase (NR) may be either cytosolic or membrane-bound (Hochstein and Tomlinson, 1988). Membrane-bound NR is generally associated with cytochrome *b* and functions in nitrate respiration, whereas cytosolic NR functions exclusively in nitrate assimilation (Hochstein and Tomlinson, 1988). Although the NR(s) of vent symbionts has not been characterized, there is evidence for nitrate respiration by symbionts of *R. pachyptila* (Hentschel and Felbeck, 1993) and lucinid clams (Hentschel et al., 1993; Barnes, 1993). For ammonia assimilation, glutamine synthetase (GS) and glutamate dehydrogenase (GDH) catalyze the formation of glutamine and glutamate from ammonia and are found in both prokaryotes and eukaryotes (Reitzer and Magasanik, 1987). GS has a higher affinity for ammonia than does GDH, with a K_m in the micromolar range compared with a K_m in the millimolar exhibited by GDH (Reitzer and Magasanik, 1987). Consequently, GS is the primary ammonia assimilation enzyme of bacteria, while GDH may be important under conditions of high ammonia availability (Stewart et al., 1980).

Some aspects of nitrogen assimilation may be mediated by the host since GS and GDH are also found in animals (Bender, 1985), including marine invertebrates (Bishop et al., 1983). In contrast, NR is restricted to organisms that respire or assimilate nitrate (Campbell and Kinghorn, 1990) and is generally believed to be absent in metazoans. Felbeck et al. (1981) showed that NR is undetectable in non-symbiotic marine invertebrates. Since inorganic nitrogen enters the organic nitrogen pool at the level of ammonia assimilation, host *versus* symbiont control of assimilation is potentially critical in understanding nutritional interactions in these mutualistic symbioses. In algal–invertebrate symbioses, host GS appears to be involved in ammonia assimilation. The expression of host GS activity in symbiotic cnidarians and giant clams is regulated by ammonia, and activity is inversely correlated with ammonia excretion (Rees, 1987; Rees et al., 1994; Yellowlees et al., 1994). Studies using the GS inhibitor methionine sulfoximine (MSX) have shown that ammonia uptake is repressed in green hydra when host GS is inhibited (McAuley, 1995). Thus, ammonia assimilation by the host may be a means of maintaining autotrophic symbionts in a nitrogen-limited state.

Distinct forms of host and symbiont GS are probably present in chemoautotrophic symbioses. Free-living bacteria express a dodecameric GS, termed GSI, consisting of identical subunits encoded for by the gene *glnA* (Brown et al., 1994). Eukaryotes express an octomeric protein, termed GSII. GSI has never been observed in eukaryotes, but *Streptomyces* spp. and some species of root nodule bacteria (rhizobia, *Frankia* sp.) have both forms (e.g. Kumada et al., 1990). GSI and GSII exhibit amino acid sequence similarity in five conserved regions associated with the catalytic active site, but sequence similarity outside these regions is low. The subunit size, thermal stability and mechanisms of post-translational regulation vary between the two types of GS (Merrick and Edwards, 1995). Since most intracellular chemoautotrophic symbionts are gamma Proteobacteria (based on an analysis of 16S rRNA sequences; Cavanaugh, 1994), they probably express a single GSI like other gamma Proteobacteria such as *Escherichia coli*.

To understand how inorganic nitrogen is utilized in support of autotrophy, the pathways of nitrogen assimilation were investigated in chemoautotrophic symbioses. The enzymatic potentials for nitrate reduction and ammonia assimilation were assessed by measuring NR, GS and GDH activities in tissue extracts from the major hydrothermal vent symbioses and from the coastal symbiotic clam *Solemya velum*. Given that activity measurements are not sufficient to resolve ammonia assimilation between the two partners, a combination of anion-exchange chromatography, immunoreactivity and thermal stability measurements was used to distinguish between host and symbiont GS protein expression in these symbioses.

Materials and methods

Collection and processing of specimens

Riftia pachyptila, *Tevnia jerichonana*, *Calyptogena magnifica* and *Bathymodiolus thermophilus* were collected using the DSV *Alvin* from a depth of 2600 m at 9°N on the East Pacific Rise. Six out of 16 *R. pachyptila*, all *T. jerichonana* and all but one *B. thermophilus* were collected during November 1994. All *C. magnifica* and the remaining *R. pachyptila* and *B. thermophilus* were collected during April 1996. Animals were brought to the surface in a thermally insulated container, then immediately placed in cold sea water (5 °C) and dissected on ice less than 1 h later. Specimens of *Solemya velum*, a shallow-living symbiotic clam, were collected in Woods Hole, MA, USA, and transported to the laboratory in sea water prior to dissection and analysis.

Symbiont-containing and symbiont-free tissues were sampled from chemoautotrophic symbioses. In the tubeworms *R. pachyptila* and *T. jerichonana*, the trophosome organ in the trunk is the symbiont-containing tissue. Samples of respiratory plume and vestimentum were collected as representative of symbiont-free tissue. Symbionts of the bivalves *C. magnifica*, *B. thermophilus* and *S. velum* are housed in the gill tissue, and the mantle or foot was sampled as a symbiont-free tissue.

The bacterium *Thiomicrospira* sp. strain L-12, isolated from deep-sea vents, was grown in artificial sea water supplemented with thiosulfate and harvested by centrifugation. *Escherichia coli* were grown overnight at 37 °C in Luria broth and harvested by centrifugation.

Enzyme activity assays

The activities of nitrogen assimilation enzymes were measured in extracts from symbiont-containing and symbiont-free tissues. Activity in symbiont-free tissues reflects host activity, whereas activity in symbiont-containing tissues is potentially the sum of host and symbiont activities. Tissue samples from freshly collected animals were minced and homogenized by hand using a ground-glass homogenizer in 3–5 volumes of ice-cold buffer containing 100 mmol l^{-1} Tris, 2.5 mmol l^{-1} $MgCl_2$ and 1 mmol l^{-1} β-mercaptoethanol at pH 7.5. Tissue homogenates were sonicated three times for 15 s, then centrifuged at 6400 revs min^{-1} in a microcentrifuge for 10 min at 4 °C. The resulting supernatants were used in measurements of enzyme activity. Protein levels in tissue extracts were determined using the bicinchoninic acid protein assay (Sigma procedure TPRO-562) using bovine serum albumin as a standard.

Glutamine synthetase transferase activity was assayed according to the method of Bender et al. (1977) in a reaction mixture containing 135 mmol l^{-1} imidazole, 18 mmol l^{-1} hydroxylamine, 0.27 mmol l^{-1} $MnCl_2$, 25 mmol l^{-1} KH_2AsO_4, 0.36 mmol l^{-1} ADP and 20 mmol l^{-1} l-glutamine, pH 7.5 at 37 °C. The reaction product, γ-glutamyl hydroxamate, was quantified spectrophotometrically at 543 nm following addition of a mixture of 0.2 mol l^{-1} $FeCl_3$, 0.12 mol l^{-1} trichloroacetic acid and 0.25 mol l^{-1} HCl. For each assay, background rates of γ-glutamyl hydroxamate production were determined in a reaction mixture without added glutamine. *E. coli* GS (Sigma Chemical Co.) was used as a positive control in all assays.

Glutamate dehydrogenase activity was assayed in the direction of glutamate formation according to the method of Murrel and Dalton (1983) in a 1 ml reaction mixture containing 50 mmol l^{-1} Tris, 5 mmol l^{-1} α-ketoglutarate, 0.25 mmol l^{-1} NADH, 25 mmol l^{-1} ammonium sulfate and 1 mmol l^{-1} ADP, pH 8 at 25 °C. The rate of NADH oxidation was determined spectrophotometrically at 340 nm. For each assay, background rates of NADH oxidation were determined in reaction mixture without added ammonium sulfate. Unless specified otherwise, NADH-dependent activities are reported. Bovine liver GDH (Sigma Chemical Co.) was used as a positive control.

Nitrate reductase activity was assayed using a protocol similar to that of Hentschel et al. (1993), which does not distinguish between dissimilatory and assimilatory NR. A reaction mixture (0.32 ml) containing 164 mmol l^{-1} phosphate, 5 mmol l^{-1} sodium nitrate, 0.2 mmol l^{-1} benzyl viologen and 2 mmol l^{-1} sodium dithionite (pH 7.4) at 20–25 °C with 1–20 µl of tissue extract was used in each assay. Production of nitrite was assessed by adding sulfanilamide and α-napthylethylenediamine to form an azo dye that was measured spectrophotometrically at 540 nm. For each assay, background rates of nitrite production were determined in reaction mixture without reduced benzyl viologen. Preliminary experiments showed that NR was not active with NADH and NADPH as electron donors (*C. magnifica* gill, *N*=5; *R. pachyptila* trophosome, *N*=8). Therefore, all reported NR activities were from sonicated samples with reduced benzyl viologen as the electron donor.

Detection of prokaryote GSI genes by DNA hybridization

The presence of prokaryote GSI genes in *R. pachyptila* and *S. velum* was tested for by Southern blotting. Genomic DNA was isolated from tissues stored at –80 °C and from *E. coli* (used as a positive control) cultured overnight in Luria broth, using a protocol developed for the preparation of genomic DNA from bacteria (Ausubel et al., 1990). Tissues or cells were lysed in a mixture of 0.5 % sodium dodecyl sulfate (SDS) and proteinase K (100 µg ml^{-1}), and the debris, polysaccharides and residual protein were then precipitated with hexadecyltrimethyl-ammonium bromide (CTAB). Genomic DNA was digested with restriction endonucleases (*Pvu*II, *Eco*RI, *Bam*HI), subjected to electrophoresis in agarose, transferred to Zeta Probe membranes (Biorad) and hybridized to labeled probe (see below) following the instructions of the manufacturer (Biorad) with a final stringency wash at 65 °C in 0.2× SSC, 0.1 % SDS (SSC is 0.032 mol l^{-1} NaCl, 0.003 mol l^{-1} sodium citrate, pH 7.0).

The coding sequence of the glutamine synthetase gene of *E.coli*, *glnA*, was used as a heterologous probe. A 1.4 kb product (the entire coding region minus 16 bases) was amplified from *E. coli* genomic DNA using the primers 5′TACTGACGATGCTGAACGAG3′ (corresponding to nucleotides +17 to +36 relative to the initiation codon) and

5′TTCCACGGCAACTAAAACAC3′ (complementary to nucleotides 1–20 downstream from the stop codon) using the polymerase chain reaction (PCR) at an annealing temperature of 60 °C and in the presence of 3 mmol l^{-1} Mg^{2+}. The PCR products were gel-purified (Qiagen Gel Extraction Kit) and used as a template to generate fluorescein-labeled probes using a random primer labeling kit (NEN-Dupont) with detection by chemiluminescence using antifluorescein–horseradish peroxidase conjugate and enhanced luminol (NEN-Dupont).

Detection of GSI expression by immunoblotting

Expression of GSI was determined by immunoblot analysis. Extracts of the tissues used in enzyme activity analyses (see above) were denatured by heating for 5 min at 100 °C in a sample buffer containing 0.125 mol l^{-1} Tris, 4 % SDS, 20 % (v/v) glycerol, 0.2 mol l^{-1} dithiothreitol and 0.02 % Bromophenol Blue, pH 6.8. Equivalent amounts of total protein were loaded on 10 % acrylamide gels and subjected to electrophoresis under denaturing conditions. Proteins were transferred from the gel and immobilized on a PVDF membrane (Millipore) by semi-dry electrophoresis according to the manufacturer's specifications (Owl Scientific). Blots were incubated with a polyclonal *E. coli* GS antiserum raised in sheep (a gift from M. T. Fisher, University of Kansas Medical Center; Fisher, 1994) or with sheep serum (Sigma Chemical Co.) as a negative control and developed by the alkaline-phosphatase-conjugated secondary antibody system according to the manufacturer's specifications (Biorad). To determine whether activity in samples is due to GSI, tissue extracts were incubated with varying amounts (0–5 µl of serum per 50 µl extract) of *E. coli* GS antiserum on ice for 1 h and then tested for GS activity as described above. Negative control experiments were conducted using sheep serum.

Separation of host and symbiont GS by anion-exchange chromatography

To determine the relative expression of eukaryote and prokaryote GS, anion-exchange chromatography was used to separate different GS forms, which were then identified on the basis of their immunoreactivity to *E. coli* GS antiserum and their thermal stability. Tissue samples stored at –80 °C were homogenized using a ground-glass homogenizer in 3–5 volumes of ice-cold grinding buffer (20 mmol l^{-1} Tris, 50 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} EDTA, 10 mmol l^{-1} β-mercaptoethanol, 1 mmol l^{-1} phenylmethylsulfonylflouride, PMSF, at pH 8.0), sonicated three times for 15 s, then centrifuged at 12 000 revs min^{-1} in a microcentrifuge for 10 min at 4 °C. The resulting supernatants were filtered through a 0.4 µm low-protein binding syringe filter (Amicon) and desalted into buffer A (20 mmol l^{-1} Tris, 10 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} EDTA, 10 mmol l^{-1} β-mercaptoethanol) using a Pharmacia biotech PD-10 column. Desalted extract was loaded onto a Pharmacia 5/5 Mono Q column and typically eluted with a 60 ml linear NaCl gradient (0–1.0 mol l^{-1}) in buffer A at a flow rate of 1 ml min^{-1} with fractions collected every minute.

The elution profile of GS activity, which could be due to host or symbiont, was determined by assaying subsamples of each fraction for GS activity as described above. Activity peaks resulting from symbiont GS were then identified on the basis of their immunoreactivity to *E. coli* GS antiserum. Subsamples (1 µl) from all collected fractions were blotted onto nitrocellulose and incubated with antiserum and developed as described above. In addition to immunoreactivity, the thermal stability of putative host and symbiont GS fractions was determined by heating subsamples at 60 °C for 10 min, which inactivates GSII but not GSI (e.g. Darrow and Knotts, 1977).

Results

Activities of inorganic nitrogen assimilation enzymes

The activities of glutamine synthetase (GS), glutamate dehydrogenase (GDH) and nitrate reductase (NR) were measured in several chemoautotrophic symbioses. NR activity was detected in tissue from *R. pachyptila*, *T. jerichonana* and *B. thermophilus*, but was negligible in *C. magnifica* and *S. velum* (Table 1). The large standard deviation in the *R. pachyptila* NR data is due to higher activity (219±106 nmol mg^{-1} min^{-1}; mean ± S.D., N=6) in worms from two dives from 1996 than in worms from other dives (24.8±14.9 nmol mg^{-1} min^{-1}; N=10). NR activity, which is not known to be present in metazoans, was indistinguishable from background in symbiont-free tissues (Table 1). NADH-dependent glutamate dehydrogenase activities, potentially of host or symbiont origin, were also measured in these symbioses (Table 1). NADPH-dependent activity was detected in *R. pachyptila*, but was 80 % lower than NADH-dependent activity. With the exception of *R. pachyptila*, where activity was greater in the vestimentum that in the trophosome, no clear differences between tissue types were observed. GS activity was detected in both symbiont-containing and symbiont-free tissues of all species tested (Table 1). Higher (P<0.05, ANOVA) activities were detected in symbiont-containing gills than in symbiont-free tissues (foot or mantle) in the coastal clam *S. velum* and the vent clam *C. magnifica*. Activities were low and similar between gill and mantle in the vent mussel *B. thermophilus*. In the tubeworms *R. pachyptila* and *T. jerichonana*, activities were higher in the symbiont-free tissues than in the samples of trophosome where the symbionts are housed. Recent measurements from *R. pachyptila* collected in 1997 indicate that trophosome GS activities can be similar to that of vestimentum in some worms (R. W. Lee and J. J. Childress, unpublished observations). In terms of host and symbiont GS and GDH, activities in symbiont-free tissues demonstrate that host activity is present, while activities in symbiont-containing tissues may reflect host and/or symbiont enzymes.

Detection of prokaryote GSI genes by DNA hybridization

Southern hybridizations, used to test for the presence of prokaryote GSI genes in symbiont-containing tissues,

Table 1. *Activities of glutamine synthetase, glutamate dehydrogenase and nitrate reductase in tissues of chemoautotrophic symbioses*

Species/Tissue	Symbionts (+/–)	Activity (nmol mg^{-1} protein min^{-1})		
		Glutamine synthetase	Glutamate dehydrogenase*	Nitrate reductase
Riftia pachyptila				
Trophosome	+	45±39 (16)	10±0.7 (7)	98±115 (16)
Vestimentum	–	289±146 (8)	43±13 (5)	0.1±0.3 (15)
Tevnia jerichonana				
Trophosome	+	3±2 (3)	ND	11.8±2.5 (3)
Plume + vestimentum	–	31±21 (3)	ND	2.4±2.5 (3)
Calyptogena magnifica				
Gill	+	81±49 (6)	2±1 (5)	0.2±0.2 (6)
Mantle	–	12±26 (5)	4±2 (5)	0.1±0.1 (5)
Bathymodiolus thermophilus				
Gill	+	16±7 (7)	3±5 (5)	4±3 (7)
Mantle	–	39±14 (6)	8±2 (3)	0.1±0.2 (6)
Solemya velum				
Gill	+	290±106 (16)	11±0.5 (9)	0.1±0.5 (18)
Foot	–	44±17 (15)	18±10 (8)	ND

Values given as mean ± S.D. (*N*), where *N* is the number of individuals from which tissue samples were collected and assayed.
ND, not determined.
*NADH-dependent activity.

indicated that a prokaryote GS gene similar to GSI of free-living gamma Proteobacteria is present in symbiont-containing tissues (Fig. 1). A heterologous probe consisting of *glnA* from *E. coli* hybridized to genomic DNA isolated from symbiont-containing tissues (trophosome and gill, respectively) of *R. pachyptila* and *S. velum*, but not to equivalent amounts of DNA from symbiont-free tissues (vestimentum and foot, respectively; Fig. 1). The stringency conditions used in the final washes should theoretically allow less then 14 % base mismatch (Sambrook et al., 1989), indicating that the probe hybridized to sequences of high identity to *E. coli glnA*. These results indicate that the chemoautotrophic symbionts have a gene encoding a GSI.

Detection of GSI expression by immunoblotting

To determine whether a GSI is expressed, the immunoreactivity of proteins from these tissues to a serum against *E. coli* GS was tested. Immunoreactive polypeptides were detected in immunoblots of total protein from all four chemoautotrophic symbioses tested and only in symbiont-containing tissues (Fig. 2). No cross-reactivity was observed in symbiont-free tissues. In all species, only one cross-reacting band was found with an estimated mobility corresponding to a molecular mass of 65–70 kDa. Subunits of *E. coli* GS exhibited an apparent molecular mass of 65 kDa (Fig. 2). Sheep serum did not cross-react with any of the samples or with *E. coli* GS. The findings that *E. coli* GS antiserum recognized a single peptide of similar size to *E. coli* GS subunits and that reactivity was only observed in symbiont-containing tissues indicate that a GSI similar to *E. coli* GS was specifically recognized by the antiserum. Although not specifically localized to the symbionts, this GSI is not likely to be of host origin and will be referred to as symbiont GSI. In subsequent experiments, immunoreactivity to *E. coli* GS antiserum was considered to be diagnostic of symbiont GSI.

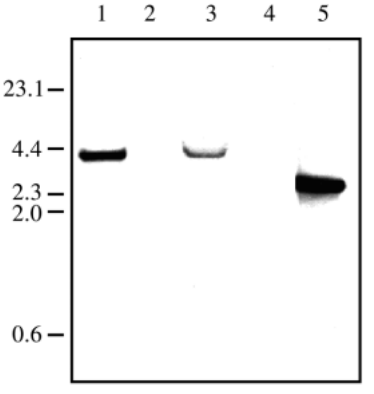

Fig. 1. Southern hybridization analysis of genomic DNA using *Escherichia coli glnA* as a probe. The blot is from a gel loaded with restriction-endonuclease-digested DNA of (1) *Solemya velum* gill, (2) *S. velum* foot, (3) *Riftia pachyptila* trophosome, (4) *R. pachyptila* vestimentum and (5) *Escherichia coli*. *S. velum* DNA (9 µg per lane) was digested with *Eco*RI, *R. pachyptila* DNA (9 µg lane^{-1}) was digested with *Bam*HI, and *E. coli* DNA (1 µg) was digested with *Pvu*II. Numbers on the left represent molecular sizes (in kilobases) obtained from labeled fragments of lambda phage digested with *Hin*dIII.

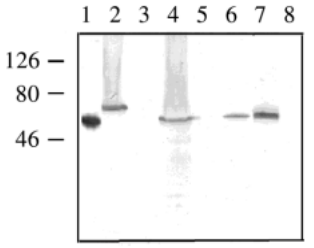

Fig. 2. Immunoblot analysis of total protein with *Escherichia coli* glutamine synthetase (GS) antiserum. The blot is from a gel loaded with (1) *E. coli* GS (Sigma Chemical Co.), and protein (100 μg per lane) extracted from (2) *Solemya velum* gill, (3) *S. velum* foot, (4) *Calyptogena magnifica* gill, (5) *C. magnifica* mantle, (6) *Bathymodiolus thermophilus* gill, (7) *Riftia pachyptila* trophosome and (8) *R. pachyptila* vestimentum. Numbers on the left represent molecular masses (in kDa) obtained from prestained molecular mass markers (Gibco).

Immunoprecipitation of GS activity

Inhibition of GS activity by addition of *E. coli* antiserum was used as a test of whether activity in these tissues is the result of the presence of GSI. Exposure to antiserum reduced GS activity of *R. pachyptila* trophosome extracts and purified *E. coli* GS (Fig. 3A). GS activity in *R. pachyptila* vestimentum, which should not contain a prokaryote GSI, was not inhibited by the antiserum (Fig. 3A). These findings indicate that GS activity in *R. pachyptila* trophosome is due, at least in part, to symbiont GSI and that activity in vestimentum is not. Exposure to sheep serum did not affect activity in extracts of *E. coli* GS or *R. pachyptila* trophosome and vestimentum (Fig. 3B). Inhibition of GS activity in *S. velum* gill extracts by *E. coli* GS antiserum was negligible, suggesting that the majority of activity in *S. velum* gill is not due to symbiont GSI.

Separation of host and symbiont GS by anion-exchange chromatography

The relative expression of host and symbiont GS was determined. Anion-exchange chromatography of total protein extracts resulted in the partial purification of two distinct GS types present in each symbiosis (Figs 4, 5; Table 2). Host GS appeared as a single peak of activity in symbiont-free tissues that was not recognized by *E. coli* GS antiserum and was thermally inactivated at 60 °C. Symbiont GSI appeared as a later-eluting peak that was immunoreactive with *E. coli* GS antiserum and thermally stable.

Two forms of GS were observed in tissues of *R. pachyptila*. Host GS was detected only in symbiont-free vestimentum tissue and eluted as a single peak at 0.1 mol l^{-1} NaCl (Fig. 4A). None of the fractions collected from vestimentum exhibited immunoreactivity with *E. coli* GS antiserum (Fig. 4A). Activity in fractions eluting at 0.1 mol l^{-1} NaCl was completely inactivated after heat treatment at 60 °C (Table 2). These findings indicate that host GS elutes as a single peak and is a eukaryote GSII. In symbiont-containing trophosome, the early-eluting peak,

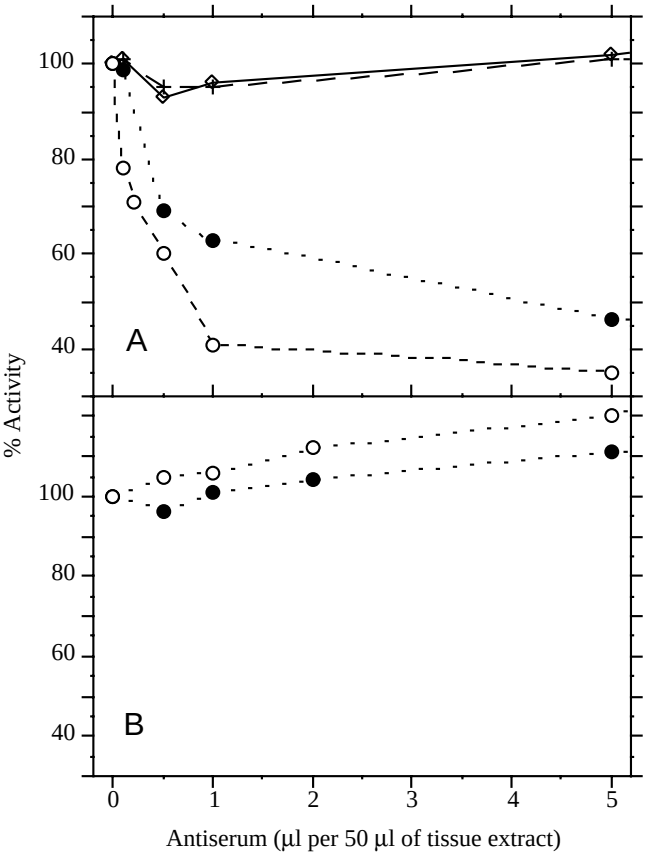

Fig. 3. Inhibition of glutamine synthetase (GS) activity in tissue extracts of *Riftia pachyptila* and *Solemya velum* by *Escherichia coli* GS antiserum. (A) Inhibition experiments using various concentrations of *E. coli* GS antiserum. Diamonds, *S. velum* gill; filled circles, *R. pachyptila* trophosome; plus signs, *R. pachyptila* vestimentum; open circles, *E. coli* GS from Sigma Chemical Co. (B) Negative control experiment using sheep serum. Filled circles, *R. pachyptila* trophosome; open circles, *E. coli* GS (Sigma Chemical Co.).

corresponding to the host GS observed in vestimentum, was not detected. The single peak of activity that was detected exhibited a different elution profile (Fig. 4B). Fractions associated with this activity peak exhibited immunoreactivity to *E. coli* GS antiserum (Fig. 4B) and were not inactivated by heating to 60 °C. Analyses with different samples of trophosome were conducted twice more with the same result (data not shown). Immunoblots of these fractions (data not shown) confirmed that the polypeptide recognized in these fractions is the same size as the immunoreactive polypeptide observed in immunoblots of total protein (see Fig. 2). These findings indicate that the predominant GS activity in trophosome is symbiont GSI.

The elution profiles and characteristics of GS from the free-living vent bacterium *Thiomicrospira* sp. strain L-12 also were determined. A single peak of activity was found to elute at high salt concentrations. The partially purified GS was thermally stable and immunoreactive with *E. coli* GS antiserum

(Table 2). These results provide further evidence that the GS activity observed in *R. trophosome* is symbiont GSI.

Symbiont GSI also predominated in symbiont-containing gill tissue of the vent bivalve symbioses *C. magnifica* and *B. thermophilus*. A large peak of activity from gill chromatography was immunoreactive with *E. coli* GS antiserum and was not inactivated by heating to 60 °C (Table 2), indicating that this late-eluting peak is symbiont GSI. Unlike *R. pachyptila*, host activity appeared to be detectable in gill tissue since a small peak of activity eluting with the same profile as host GS in foot tissue was observed (data not shown). Like *R. pachyptila*, host GS in symbiont-free foot tissue of both species eluted earlier than symbiont GSI in gill tissue, did not cross-react with *E. coli* GS antiserum and was completely inactivated by treatment at 60 °C (Table 2).

In contrast to the other chemoautotrophic symbioses, symbiont GSI protein, but not activity, was detected in *S. velum*. Fractions eluting at the same NaCl concentrations as symbiont GS from *C. magnifica* and *B. thermophilus* were immunoreactive with *E. coli* GS antiserum but did not exhibit GS activity (Fig. 5; Table 2). Additional analyses conducted on gills from freshly collected *S. velum* yielded the same result. Immunoreactivity results give a measure of presence or absence but not of quantity. Thus, symbiont GSI was present in *S. velum* gill, but may have been present at too low a level to exhibit detectable activity or may have been inactivated.

The activity that was detected in *S. velum* gill may be host GS. Host GS in *S. velum* foot eluted early, was not immunoreactive to *E. coli* GS antiserum and was inactivated by treatment at 60 °C (Fig. 5A; Table 2). The single peak of activity observed in runs of symbiont-containing gill tissue exhibited identical characteristics to host GS from foot (Fig. 5B; Table 2).

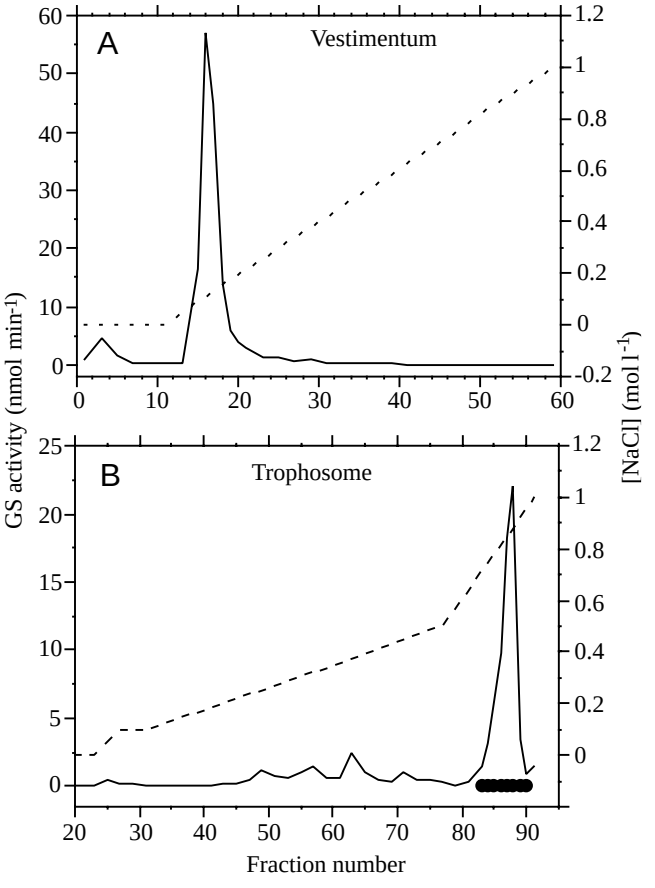

Fig. 4. Elution profile of *Riftia pachyptila* glutamine synthetase (GS) from anion-exchange chromatography runs. Solid lines, GS activity; dashed lines, NaCl concentration in buffer gradient; filled circles in B denote fractions that were immunoreactive with *Escherichia coli* GS antiserum. (A) *R. pachyptila* vestimentum; (B) *R. pachyptila* trophosome.

Discussion

In the present study, the activities of key assimilation enzymes were determined, and host and symbiont GS were distinguished. Measurements of GS, GDH and NR activity indicated that key pathways involved in the assimilation of ammonia and nitrate by free-living autotrophs are present in chemoautotrophic symbioses, but are not sufficient to differentiate between host and symbiont expression. To investigate the question of whether the host or the symbiont is responsible for ammonia assimilation, levels of expression of

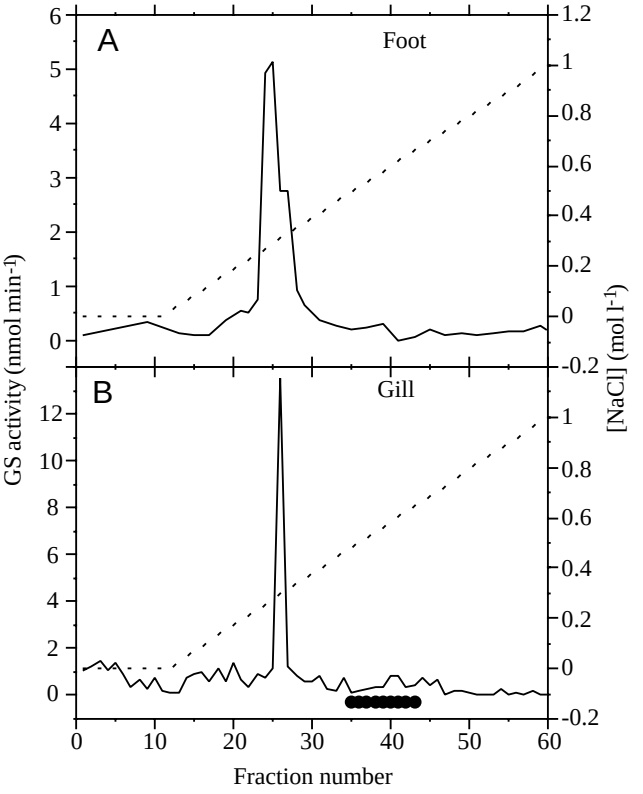

Fig. 5. Elution profile of *Solemya velum* glutamine synthetase (GS) from anion-exchange chromatography runs. Solid lines, GS activity; dashed lines, NaCl concentration in buffer gradient; filled circles in B denote fractions that were immunoreactive with *Escherichia coli* GS antiserum. (A) *S. velum* foot; (B) *S. velum* gill.

Table 2. *Characteristics of partially purified glutamine synthetase from anion-exchange chromatography fractions*

Sample	Tissue type	α-GSI immunoreactivity	GS activity after 60 °C treatment (%)	Putative type
Chemoautotrophic symbioses				
Riftia pachyptila				
Trophosome	Symbiont-containing	Yes	96, 86	GSI
Vestimentum	Symbiont-free	No	1	GSII
Calyptogena magnifica				
Gill	Symbiont-containing	Yes	87	GSI
Foot	Symbiont-free	No	0.3	GSII
Bathymodiolus thermophilus				
Gill	Symbiont-containing	Yes	84	GSI
Foot	Symbiont-free	No	0.5	GSII
Solemya velum				
Gill (fraction 26)	Symbiont-containing	No	22	GSII
Gill (fractions 37–41)	Symbiont-containing	Yes	NA	GSI
Foot	Symbiont-free	No	21	GSII
Free-living bacterium				
Thiomicrospira sp. strain L-12		Yes	97	GSI
Controls				
GSI: *E. coli* GS		Yes	96	
GSII: Sheep brain GS		No	4	

Immunoreactivity to α-GSI antiserum and sensitivity of activity to 60 °C treatment (diagnostic of GSII) was tested in fractions from which the highest glutamine synthetase activity was observed.

Escherichia coli GS and sheep brain GS were purchased from Sigma Chemical Co.

NA, no activity detected; GS, glutamine synthetase.

GS activity after heat treatment is given as a percentage of the value before treatment.

host and symbiont GS in symbiont-containing tissue, where nitrate and ammonia are assimilated (Lee and Childress, 1994), were also determined. The results of the present study show that a prokaryote GS is the predominant GS expressed in symbiont-containing tissues of symbioses from hydrothermal vents. This GS is apparently symbiont GSI. In contrast, GSI activity was not detected in symbiont-containing tissue of *S. velum*. Instead, a single peak of activity was observed that appears to be host GS.

Nitrate reductase

Nitrate is the most abundant inorganic source available to vent symbioses at unsedimented ridges (Johnson et al., 1988; Lee and Childress, 1994). In contrast, for symbioses living in reducing sediments such as *S. velum*, ammonia is a more abundant inorganic source than nitrate (Lee and Childress, 1994). Thus, for vent symbioses in particular, nitrate reduction catalyzed by NR is necessary for growth on inorganic nitrogen.

Nitrate reductase is almost certainly localized to the symbionts, but may have dual functions. Nitrate can be used by bacteria as an alternative electron acceptor as well as a potential nitrogen source. Nitrate respiration has been proposed in several chemoautotrophic symbioses including *R. pachyptila* (Hentschel and Felbeck, 1993) and the sediment-dwelling clams *Solemya reidi* (Wilmot and Vetter, 1992) and *Lucinoma aequizonata* (Hentschel et al., 1993). Whether NR activities measured in the present study were due to soluble assimilatory NR, membrane-bound dissimilatory NR or both is not known. If the NR activity of the symbionts is of a dissimilatory type, it may have a role in nitrate assimilation if the nitrite produced is reduced to ammonia and assimilated rather than lost by excretion of nitrite or by reduction of nitrite to NO_x or N_2 (Cole, 1988).

To support nitrate assimilation, NR activities are likely to be higher in vent symbioses than in *S. velum* since nitrate is abundant at vents. With the exception of *C. magnifica*, which showed negligible NR activity, this appears to be the case (Table 1). Alternatively, elevated NR activity in tubeworms may reflect the utilization of nitrate in respiration. The low NR activity in *C. magnifica* is inconsistent with growth based on nitrate. One explanation is that the *C. magnifica* collected for this study were not growing autotrophically. Variation in NR activity merits further investigation. Since hydrothermal vents communities are ephemeral, with rapid growth and colonization followed by senescence and death (Lutz and Kennish, 1993), the loss of NR activity could indicate that animals are switching from autotrophy to heterotrophy. Indeed, *R. pachyptila* collected away from active venting do not exhibit detectable NR activity (R. W. Lee and C. M. Cavanaugh, unpublished results).

The symbiont NR activities measured in this study are within the range for free-living bacteria. Since NR activity

should be localized to the symbionts, activities expressed per milligram of total protein are underestimates of activity per milligram of symbiont protein. According to Powell and Somero (1986), 15–35 % of *R. pachyptila* trophosome and 4.0 % of *B. thermophilus* gill volume is made up of symbionts. Since symbiont NR activity ≈ (activity in symbiont-containing tissue/percentage of symbionts)×100, *R. pachyptila* and *B. thermophilus* therefore have estimated symbiont NR activities of 283–653 nmol mg^{-1} min^{-1} and 88 nmol mg^{-1} min^{-1} respectively. *E. coli*, which has a membrane-bound respiratory NR and can only grow on nitrate under anaerobic conditions (Stewart, 1994), exhibits NR activity ranging from 0 (under aerobic conditions or without nitrate present) to 1400 nmol mg^{-1} min^{-1} (anaerobic, nitrate present; Showe and DeMoss, 1968). *Klebsiella aerogenes*, which has soluble and membrane-bound forms of NR and can assimilate nitrate under both anaerobic and aerobic conditions (Cole, 1988), exhibits NR activity (with nitrate as the sole nitrogen source) of 2–10 nmol mg^{-1} min^{-1} when grown aerobically and 350–800 nmol mg^{-1} min^{-1} when grown anaerobically (Van't Riet et al., 1968). The NR activity of *Beggiatoa alba* (a free-living sulfur-oxidizing bacterium), which has a soluble NR and cannot respire nitrate, ranges from 122 to 138 nmol mg^{-1} min^{-1} (Vargas and Strohl, 1985). On the basis of activity measurements, it would appear that there is sufficient NR activity in vent tubeworms and *B. thermophilus* for either assimilation or respiration of nitrate.

Further characterization of symbiont NR, nitrite reductase and the products of nitrite reduction are needed. From the results presented here, it is possible to conclude that *R. pachyptila, T. jerichonana* and *B. thermophilus* have a high to moderate NR activity that is consistent with the utilization of nitrate as a nitrogen source, as demonstrated previously (Lee and Childress, 1994). Nitrate assimilation may be low in some *C. magnifica* and in *S. velum* living in reducing sediments.

Glutamate dehydrogenase

Inorganic nitrogen first enters the organic pool *via* the assimilation of ammonia into glutamine and glutamate. In the case of vent symbioses, ammonia is probably derived from nitrate reduction and in *S. velum* from environmental uptake. Ammonia assimilation may be catalyzed by host or symbiont GDH. Since GDH has a high K_m for ammonia (for a review, see Stewart et al., 1980), this is consistent with the finding of elevated ammonia levels in tissues of chemoautotrophic symbioses (Lee and Childress, 1996; Lee et al., 1997). However, GDH activities of chemoautotrophic symbioses are low, with the exception of *R. pachyptila* vestimentum. The non-symbiotic bivalves *Geukensia demissa* and *Mytilus edulis* exhibit GDH activities of 55 and 30 nmol mg^{-1} min^{-1} respectively (Reiss et al., 1977; Livingstone, 1975), and the sipunculid *Phascolosoma arcuatum* exhibits an activity of 15 nmol mg^{-1} min^{-1} (Ip et al., 1994). In algal–invertebrate associations, GDH activity is undetectable in host tissue of *Tridacna gigas* (Rees et al., 1994), but is similar to that of non-symbiotic invertebrates in *Pocillopora damicornis* (Yellowlees et al., 1994) and green hydra (Rees, 1987). These results suggest that the potential for ammonia assimilation by GDH is not enhanced in chemoautotrophic symbioses compared with non-symbiotic invertebrates that do not assimilate ammonia in support of growth. Thus, GDH does not appear to be involved in assimilation, although further investigation is needed to rule this out.

Glutamine synthetase

Glutamine synthetase is the primary enzyme catalyzing ammonia assimilation in bacteria and plants (Reitzer and Magasanik, 1987). In chemoautotrophic symbioses, GS is potentially of either host or symbiont origin, and thus the involvement of host *versus* symbiont GS in assimilation in symbiont-containing tissues is ambiguous. A major result of the present study was the quantification of host and symbiont GS activity using a combination of anion-exchange chromatography, immunoreactivity and thermal stability measurements.

Prokaryote GSI was detected in symbiont-containing tissues and is unlikely to be of host origin. GSI is considered to be a prokaryote enzyme, and neither genes encoding GSI nor GSI expression were detected in symbiont-free tissue (Figs 1, 2). Thus, we conclude that this GSI is symbiont GSI. Since the regulation of GSI in free-living bacteria has been well characterized (for a review, see Merrick and Edwards, 1995), it would be of interest to determine whether similar regulatory mechanisms function in chemoautotrophic symbioses. While fast growth may benefit free-living bacteria, symbiont growth, if uncontrolled, could be deleterious in a mutualistic symbiosis.

Symbiont GSI accounted for all, or nearly all, the GS activity in symbiont-containing tissues of the vent symbioses *R. pachyptila, C. magnifica* and *B. thermophilus*. Thus, in the symbiont-containing tissues, the conversion of nitrate and ammonia to organic nitrogen (at least to glutamine) appears to be localized to the symbionts. Symbiont GS activity is within the low range for free-living bacteria in *R. pachyptila* and *B. thermophilus* but not in *C. magnifica*. Using estimates based on percentage symbiont values (see above), GS activities are 131–302 nmol mg^{-1} min^{-1} for *R. pachyptila* symbionts, 1053 nmol mg^{-1} min^{-1} for *C. magnifica* symbionts and 400 nmol mg^{-1} min^{-1} for *B. thermophilus* symbionts. GS activities of 20–1500 nmol mg^{-1} min^{-1} have been measured in the free-living bacterium *Klebsiella aerogenes*, with lower activities exhibited under conditions of high nitrogen availability (Magasanik, 1982). The low symbiont activities of *R. pachyptila* and *B. thermophilus* could be related to high internal ammonia availability.

In contrast to the vent symbioses, no symbiont GSI activity was detectable in *S. velum*. Whether the absence of activity is due to a low level of GSI expression or to inhibitory factors is still an open question. These findings suggest that ammonia is assimilated in *S. velum* by a different mechanism from that in the other symbioses tested. GS activity in gill is due to host GSII or, alternatively, to a novel symbiont GSII that exhibits

similar characteristics to those of host GS. Bacterial GSII has been observed in only a few species of bacteria and is also thermally labile, but shares less than 50 % amino acid identity with eukaryote GS sequences (Carlson and Chelm, 1986). Other differences include a subunit size of 36 kDa compared with 41 kDa for invertebrate GSII (Trapido-Rosenthal et al., 1993). Whether the bacterial GSII and host GSII exhibit the same elution profile was not tested. Further tests are needed to confirm whether GS activity in gill and foot are due to the same host enzyme.

Glutamine synthetase activity in *S. velum* gill (Table 1) is higher than in non-symbiotic invertebrates. GS activities are 59–67 nmol mg^{-1} min^{-1} in the ribbed mussel *Geukensia demissa* (R. W. Lee and C. M. Cavanaugh, unpublished results), 3–20 nmol mg^{-1} min^{-1} in *Octopus vulgaris* (Kleinschuster and Morris, 1972) and 1–4 nmol mg^{-1} min^{-1} in *Cancer magister* (Kleinschuster and Morris, 1972). Thus, if the primary GS in *S. velum* gill is of host origin, the activity is higher than in non-symbiotic invertebrates, presumably to accommodate the higher metabolic demand for ammonia assimilation. In some algal–invertebrate symbioses, the hosts are thought to be involved in assimilation and exhibit activity in the same range or higher than that in *S. velum* (Rees, 1987; Yellowlees et al., 1994; Rees et al., 1994). Activities in host tissues are 1560 nmol mg^{-1} min^{-1} in the giant clam *Tridacna gigas* (Rees et al., 1994), 186 nmol mg^{-1} min^{-1} in the coral *Pocillopora damicornis* (Yellowlees et al., 1994) and 420–720 nmol mg^{-1} min^{-1} in green hydra (Rees, 1987). Higher activities in algal–invertebrate symbioses may reflect the lower environmental availability of inorganic nitrogen compared with the reducing sediment environment of *S. velum*.

If ammonia is indeed assimilated in support of growth by host GS in *S. velum*, this is a novel capability of symbiotic invertebrates. This raises the issue of whether the host can convert glutamine into other amino acids. The second step in ammonia assimilation is catalyzed by glutamate synthase (GOGAT), which is not generally believed to be present in animals, although there is a report of GOGAT activity in silkworm (*Bombyx mori*) embryos and larvae (Seshachalam et al., 1992). Further tests are needed to determine whether GOGAT activity is present in symbiotic invertebrates.

Ammonia assimilation by the host and repression of symbiont GS occur in a variety of symbioses. For example, ammonia is apparently assimilated by the host in symbioses between marine algae and invertebrates (Rees et al., 1994; McAuley, 1995) since host GS activity is responsive to ammonia availability and correlates with ammonia assimilation and uptake (Rees, 1987; Rees et al., 1994; Yellowlees et al., 1994; McAuley, 1995). In prokaryote–eukaryote associations between nitrogen-fixing rhizobia and plants, the prokaryote symbiont GS is repressed so that ammonia produced from nitrogen fixation is assimilated exclusively by the plant. In legume root nodules, more than 95 % of GS activity is in the plant cytosol, and it is the presence of the symbiont that induces increased plant GS expression (Cullimore and Bennett, 1988). Symbiont GS is also repressed in the cyanobacteria *Anabaena*–water fern symbiosis. A greater than tenfold reduction in levels of symbiont GS mRNA is observed compared with free-living *Anabaena* (Nierzwicki-Bauer and Haselkorn, 1986). Thus, it appears that assimilation by the host rather than by the symbionts occurs in diverse symbiotic associations.

Patterns of host *versus* symbiont GS expression may be related to the mechanism of nutritional transfer from symbiont to host. One mechanism involves harvesting of symbionts by digestion. In such a scenario, symbiont growth and division occur and are consistent with the expression of symbiont GS. A second mechanism involves translocation of organic compounds from symbiont to host, but the mechanisms by which the host induces the symbiont to release organic compounds are not known in chemoautotrophic symbioses. In algal–invertebrate symbioses, factors produced by the host have been shown to cause the release of organic compounds by the symbionts *in vitro* (e.g. Muscatine, 1967; Trench, 1971; Gates et al., 1995). Another way in which symbionts may be induced to release organic carbon to the host is if ammonia is shunted away from the symbionts *via* assimilation by host GS. As a result, carbon derived from CO_2 fixation cannot be converted to amino acids and may be released to the host. Thus, if transfer is by digestion, symbiont GS is expressed, whereas if transfer is by translocation, symbiont GS may be repressed. The results from the present study appear to be consistent with the mode of nutritional transfer for solemyid clams and *B. thermophilus*. In *S. reidi*, the mode of organic carbon transfer is by translocation (Fisher and Childress, 1986). A similar mechanism is probably present in *S. velum* and thus might result from host assimilation of ammonia. In *B. thermophilus*, transfer is believed to occur *via* digestion (Fiala-Médioni and Métivier, 1986), and in the present study, symbiont GS was found to predominate. Similarly, in the other species investigated, *R. pachyptila* and *C. magnifica*, the expression of symbiont GS is consistent with nutritional transfer by digestion. While the mechanism of carbon transfer in *C. magnifica* is not known, in *R. pachyptila* there is evidence for transfer by digestion since degeneration of symbionts is observed in the periphery of trophosome lobules (Bosch and Grasse, 1984; Hand, 1987).

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