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Immunochemical localization of ribulose-1,5-bisphosphate carboxylase in the symbiont-containing gills of *Solemya velum* (Bivalvia: Mollusca)

(chemoautotroph/sulfur-oxidizing bacteria/deep-sea hydrothermal vents/symbiosis)

COLLEEN M. CAVANAUGH*, MARILYN S. ABBOTT*†, AND MARTEN VEENHUIS‡

*Harvard University, The Biological Laboratories, 16 Divinity Avenue, Cambridge, MA 02138; †Anheuser-Busch Companies, Corporate Research and Development, Saint Louis, MO 63118; and ‡Laboratory of Electron Microscopy, University of Groningen, Biological Centre, Kerklaan 30, 9751 NN Haren, The Netherlands

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ABSTRACT **The distribution of the Calvin cycle enzyme ribulose-1,5-bisphosphate carboxylase (RbuP_2Case; EC 4.1.1.39) was examined by using two immunological methods in tissues of *Solemya velum*, an Atlantic coast bivalve containing putative chemoautotrophic symbionts. Antibodies elicited by the purified large subunit of RbuP_2Case from tobacco (*Nicotiana tabacum*) cross-reacted on immunoblots with a protein of similar molecular mass occurring in extracts of the symbiont-containing gill tissue of *S. velum*. No cross-reactivity was detected in symbiont-free tissue extracts. The antiserum also cross-reacted in immunoblots with proteins of *Thiobacillus neapolitanus*, a free-living sulfur-oxidizing chemoautotroph whose RbuP_2Case has been well characterized. In protein A–gold immunoelectron microscopy studies, this antiserum consistently labeled the symbionts but not surrounding host gill tissue, indicating that the symbionts are responsible for the RbuP_2Case activity.**

Increasing evidence suggests that sulfur-oxidizing chemoautotrophic bacteria occur as endosymbionts in a variety of marine invertebrates including deep-sea hydrothermal vent tubeworms and coastal sediment bivalves [reviewed by Cavanaugh (1)]. By analogy to their free-living counterparts (e.g., bacteria of the genera *Thiobacillus*), these symbionts are thought to be capable of deriving their cellular carbon from carbon dioxide and their energy from the respiration of reduced inorganic substrates such as sulfide and thiosulfate. Although free-living sulfur bacteria are well known (2), their symbiotic existence was not previously recognized. As intracellular inhabitants, chemoautotrophs could provide their host with an internal source of organic compounds and thus may play a nutritional role parallel to that of chloroplasts in plants.

Although the existence of "chemoautotrophic symbioses" is now widely accepted, the symbionts have not yet been cultured, and therefore little is known about their metabolic capabilities. Symbionts occur in certain tissues of marine invertebrates and resemble Gram-negative prokaryotic cells when observed with transmission electron microscopy. The major evidence supporting the hypothesis that they are autotrophs is the detection of ribulose-1,5-bisphosphate carboxylase (RbuP_2Case; EC 4.1.1.39) activity in those same tissues (1). Since RbuP_2Case only occurs in organisms that employ the Calvin cycle—that is, plants, green algae, cyanobacteria, most anaerobic photosynthetic bacteria, and aerobic chemoautotrophs (for reviews, see refs. 3 and 4)—it is assumed that the bacterial symbionts are responsible for the RbuP_2Case activity detected in the animal tissues. To determine if the symbionts are responsible for the RbuP_2Case activity in the *Solemya velum* clam–bacteria symbiosis, we set out to localize this enzyme in the clam tissue by immunochemical means.

The RbuP_2Case holoenzyme of vascular plants, green algae, and most bacteria is a high molecular mass complex (≈550 kDa) composed of eight large subunits and eight small subunits, with molecular masses of 50–56 kDa and 11–15 kDa, respectively (3, 4). The enzyme catalyzes both carboxylation and oxygenation reactions by using ribulose 1,5-bisphosphate as a substrate. Although the function of the small subunit (SSU) remains unknown, the large subunit (LSU) has been shown to contain the catalytic site and the CO_2/Mg^{2+} activation site of the enzyme (3).

All available evidence indicates that the RbuP_2Case LSUs of various higher plants, green algae, and cyanobacteria are similar in amino acid composition (3). Little information is available for many of the bacterial RbuP_2Cases; however, amino acid sequence similarity has been demonstrated in the LSUs of the hydrogen bacterium *Alcaligenes eutrophus* and the purple nonsulfur bacterium *Rhodospirillum rubrum* with plant-type LSUs, notably in regions of the enzyme implicated as the catalytic and activator sites (5, 6). Furthermore, recent studies employing the technique of heterologous DNA hybridization have shown that portions of the cloned RbuP_2Case LSU gene (*rbcL*) from the cyanobacterium *Anacystis nidulans* 6301 hybridize with DNA from a variety of photosynthetic and chemoautotrophic bacteria, including the thiobacilli, which implies DNA sequence similarity among these organisms (7).

Antisera directed against the LSU of RbuP_2Case have been used to localize this enzyme by immunogold labeling in the chloroplasts of *Chlamydomonas reinhardtii* (8), the photosynthetic cyanelles of *Cyanophora paradoxa* and *Glaucocystis nostochinearum* (9), and in the cyanobacteria *Chlorogloeopsis fritschii* and *Anabaena cylindrica* (10, 11). In other studies, antibodies directed against either the chloroplast or cyanobacterial RbuP_2Case LSU immunoprecipitate both proteins but generally do not precipitate proteins of other photosynthetic bacteria or chemoautotrophs (reviewed in refs. 4 and 12). However, the strong sequence similarity between the LSUs of various organisms predicts that specific binding of antibodies directed against the LSU of higher plants to the LSUs of chemoautotrophs should be detectable by using a more sensitive technique such as the immunoblot procedure.

MATERIALS AND METHODS

Organisms. *S. velum* were collected from eelgrass beds near Woods Hole, MA, and were placed in filtered (0.22 μm) seawater to cleanse body surfaces prior to dissection. Upper leaves of tobacco, *Nicotiana tabacum* var. Wisconsin 38,

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Abbreviations: RbuP_2Case, ribulose-1,5-bisphosphate carboxylase; LSU, large subunit; TLSU, tobacco LSU.

were harvested from greenhouse-grown plants. *Thiobacillus neapolitanus* (strain X), a free-living sulfur-oxidizing chemoautotroph whose RbuP_2Case has been well characterized, was grown in a 1-liter chemostat under CO_2 limitation with 40 mM thiosulfate as an energy source, as described by Holthuijzen *et al.* (13). The overflow of the chemostat was stored at 4°C until harvesting of the cells.

Enzyme Purification and Preparation of Antiserum. RbuP_2Case was isolated from leaves of *N. tabacum* as described by Poulsen (14). The individual polypeptide subunits were separated by sodium dodecyl sulfate (SDS)/PAGE (15). Gel-purified tobacco LSU (TLSU) was used to produce antibodies in a rabbit. Preimmune serum was collected from the animal before the first immunization. The specificity of the antiserum was tested by immunoblot analysis of a crude extract of *N. tabacum* soluble leaf protein. Only a single band, comigrating with RbuP_2Case LSU, was recognized by the antiserum (see Fig. 1*A*, lane 1).

Gel Electrophoresis and Immunoblotting. Extracts of *S. velum* tissues, *N. tabacum* leaves, and *T. neapolitanus* cells were prepared for gel electrophoresis as follows. *S. velum* gill tissues (gills, endosymbionts, and attached hypobranchial gland) and foot tissues (foot with internal gonad tissue) were dissected from freshly collected clams, weighed, frozen in liquid nitrogen, and stored at −80°C. Frozen tissues were homogenized in 10–25 volumes of Tris-buffered saline (40 mM Tris/0.9% NaCl, pH 7.5) in ground glass tissue grinders on ice. *N. tabacum* leaves were thoroughly ground in Tris-buffered saline (0.2 g of fresh weight per ml of buffer) with an Omni mixer (Omni, Waterbury, CT). The soluble extract, obtained after centrifugation at 12,000 × *g* for 10 min, was stored frozen at −20°C. *T. neapolitanus* cells were harvested by centrifugation (10,000 × *g* for 10 min at 4°C), resuspended in 10 mM Hepes-KOH, pH 8.0/20 mM $MgCl_2$/1 mM dithiothreitol/2 mM phenylmethylsulfonyl fluoride, a serine protease inhibitor (13), and were stored frozen at −80°C.

Prior to gel electrophoresis, subsamples of tissue and cell homogenates were diluted with sample buffer to the following final concentrations: 0.0625 M Tris·HCl (pH 6.8), 6.25% glycerol, 3% SDS, 0.05 M dithiothreitol, and 0.0125% bromophenol blue. The samples were heated at 100°C for 5 min and then were centrifuged (12,000 × *g* for 5 min). Ten to 100 μg of total protein were loaded per lane.

Proteins were separated by SDS/PAGE by using 12% gels, essentially according to Laemmli (15). For immunoblot analysis, proteins were electrophoretically transferred to nitrocellulose at 50 V for 2 hr at 4°C in buffer containing 25 mM Tris base, 192 mM glycine, and 0.01% SDS. Immunostaining of the protein blots was carried out by using the avidin–biotin–peroxidase complex method according to manufacturers instructions (Vector Laboratories, Burlingame, CA). The anti-TLSU serum was used at a 1:500 dilution. Control blots were prepared by using preimmune serum at the same dilution. Proteins in replicate gels were stained with Coomassie blue.

Electron Microscopy and Immunocytochemistry. *S. velum* gills were dissected, fixed with 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 60 min at 0°C, dehydrated in a graded ethanol series, and embedded in Lowicryl K4M (16). Immunogold labeling of RbuP_2Case was performed on ultrathin sections by the method of Slot and Geuze (17). Gold particles of 8 nm were prepared by the citrate method of Frens (18). In control experiments, preimmune rabbit serum or anti-TLSU serum treated with spinach RbuP_2Case (Sigma) to preabsorb anti-TLSU IgG were used in place of the anti-TLSU serum.

RESULTS

Specificity of the Antiserum. To determine if antiserum directed against the LSU of RbuP_2Case from tobacco cross-reacts with polypeptides from symbiotic or free-living chemoautotrophs, whole extracts of *S. velum* tissues and *T. neapolitanus* (Fig. 1) were examined by the immunoblot procedure. Although numerous protein bands were evident in *S. velum* tissue extracts stained with Coomassie blue (Fig. 1*A*), anti-TLSU serum cross-reacted with only one protein band in all of the symbiont-containing gills of *S. velum* tested (Fig. 1*B*, lanes 2–4). Two discrete bands were visible in the *T. neapolitanus* extract. The cross-reactive bands in each lane have approximately the same relative mobility as tobacco RbuP_2Case LSU (≈55 kDa). No antiserum binding was detected in extracts of *S. velum* foot and gonad tissues (Fig. 1*B*, lanes 5–7). Cross-reactivity was not observed with any of the extracts when preimmune serum was substituted for anti-TLSU (data not shown).

Localization of RbuP_2Case in *S. velum*. Numerous bacterial symbionts were observed in the gill tissue of *S. velum* with

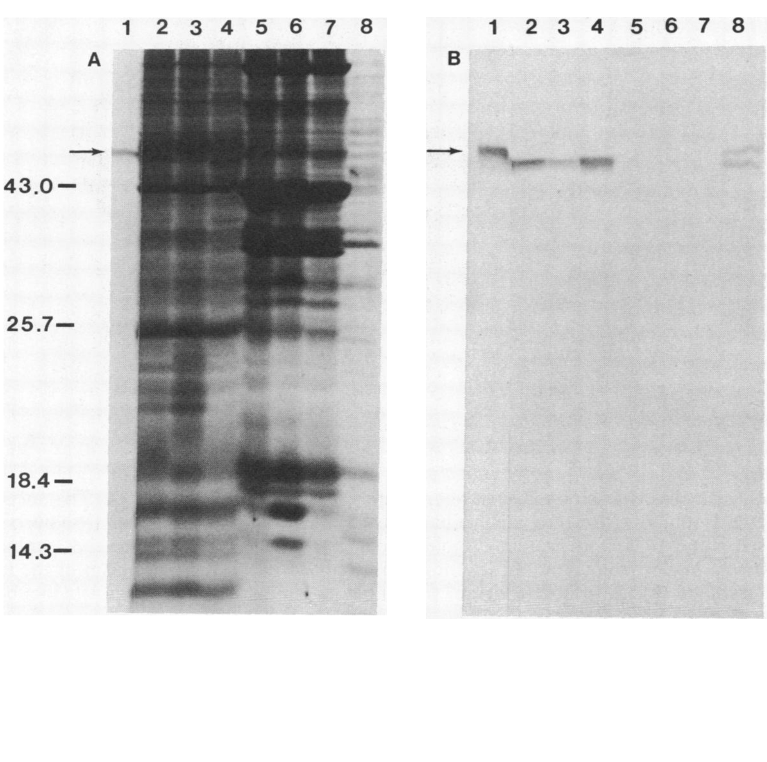

FIG. 1. (*A*) SDS/PAGE of proteins from *N. tabacum* (lane 1), *S. velum* symbiont-containing gill tissues (lanes 2–4), *S. velum* combined foot and gonadal tissues containing eggs (lanes 5 and 7) or sperm (lane 6), and *T. neapolitanus* (lane 8). Lane 1 was loaded at a concentration at which the LSU of *N. tabacum* Rbu-P_2Case was essentially the only protein that stained with Coomassie blue. (*B*) Immunoblot analysis of gel as in *A* incubated with anti-TLSU serum. →, Position of *N. tabacum* RbuP_2Case LSU (55 kDa). Numbers represent molecular size standards: ovalbumin (43 kDa), chymotrypsinogen (25.7 kDa), lactoglobulin (18.4 kDa), and lysozyme (14.3 kDa).

transmission electron microscopy (Fig. 2). As described previously (19), they are intracellular—contained within membrane-bound vacuoles of bacteriocytes (gill epithelial cells). After incubation with the anti-TLSU serum and protein A–gold, specific labeling was observed only on profiles of the bacterial symbionts (Fig. 2*A*). This pattern was identical in all *S. velum* gill tissues examined. Host cell cytoplasm, membranes, and other organelles, such as mitochondria, were not labeled. The gold particles were evenly distributed over the symbiont cytoplasm but were not associated with the cell envelope, nuclear region, or storage granules (Fig. 2*B*). No significant labeling was observed on control sections treated with preimmune serum (Fig. 2*C*) or with anti-TLSU serum pretreated with spinach RbuP_2Case (data not shown).

DISCUSSION

Antiserum directed against tobacco RbuP_2Case LSU cross-reacted with proteins in extracts of both free-living and symbiotic chemoautotrophs. Since this antiserum has been shown to be specific for RbuP_2Case LSU in tobacco extracts, we postulate that anti-TLSU serum recognized the LSU of RbuP_2Case present in both *S. velum* gills and *T. neapolitanus*. This hypothesis is based on the following observations: (*i*) the antibody cross-reacted with proteins having mobilities similar to those reported for bacterial RbuP_2Case LSUs, (*ii*) the antibody cross-reacted with these proteins only in tissues of *S. velum* having detectable RbuP_2Case activity [i.e., the symbiont-containing gills but not foot tissues (19)], (*iii*) gold-particle labeling of thin sections is abolished in competitive inhibition studies with anti-TLSU serum pretreated with purified spinach RbuP_2Case, and (*iv*) preimmune serum showed no cross-reactivity with any of the cell or tissue extracts. Thus, the anti-LSU labeling appeared to be specific for the large subunit of RbuP_2Case in both *S. velum* and *T. neapolitanus*.

The presence of two labeled bands in extracts of *T. neapolitanus* may have been caused by endogenous heterogeneity of this enzyme, or it may have been due to specific degradation of the LSU. RbuP_2Case of *T. neapolitanus* has been characterized biochemically (13, 20, 21), and the holo-

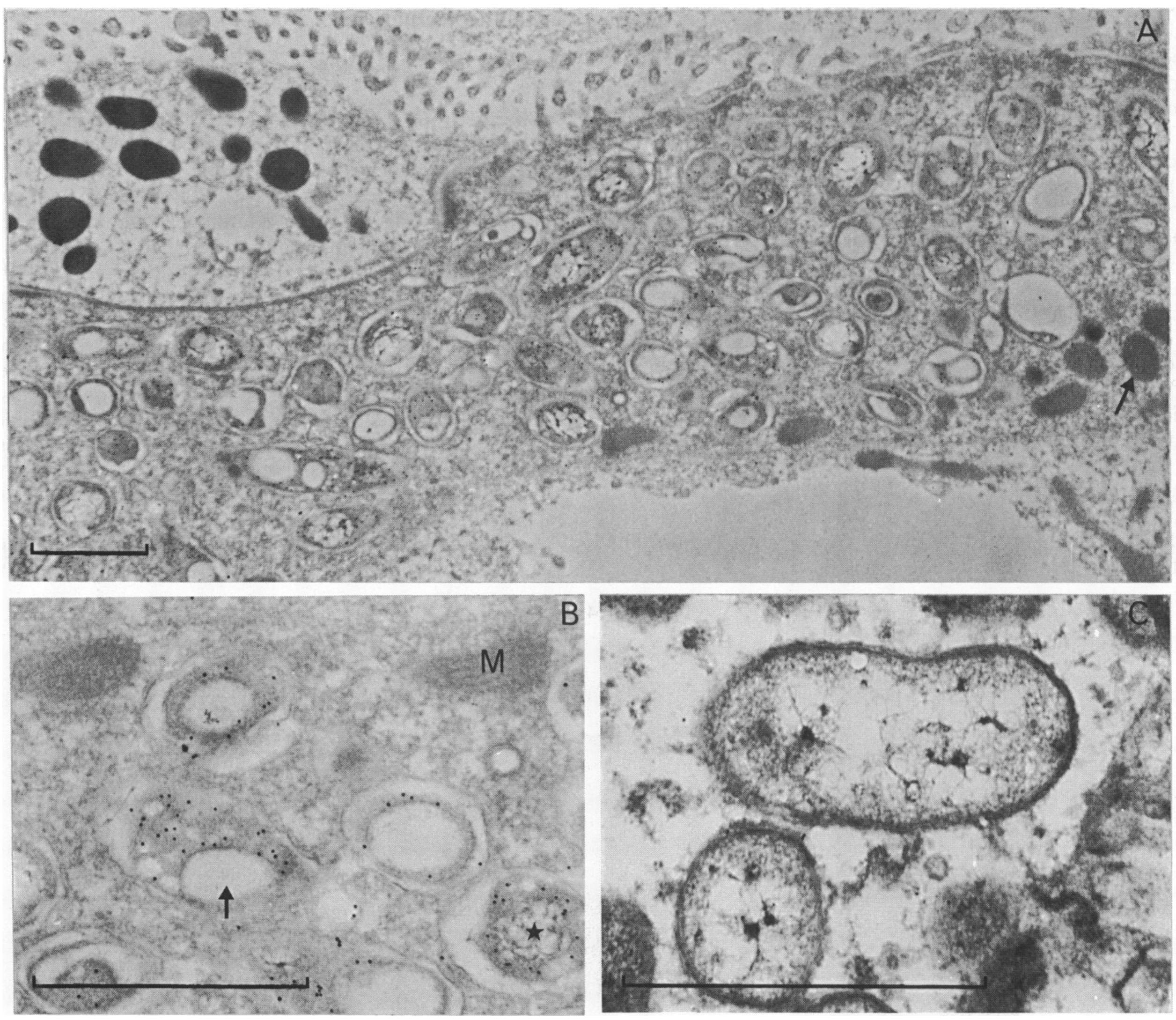

FIG. 2. Immunocytochemical localization of RbuP_2Case. Transmission electron micrographs of *S. velum* gill tissue. (*A*) The survey shows a portion of a bacteriocyte containing numerous bacterial symbionts, flanked by a symbiont-free cell fringed with microvilli (top portion). After incubation of ultrathin sections with antiserum against RbuP_2Case and protein A–gold, specific labeling is confined to the symbionts; cell organelles like mitochondria (arrow) are unlabeled. (*B*) At higher magnification, labeling is only observed on the cytoplasm of the symbionts but is lacking on the nuclear region (★) and storage granules (♠). M, mitochondrion. (*C*) Labeling is also absent in controls incubated with preimmune serum. (Bar = 1 μm.)

enzyme appears to be uniform. However, these workers have reported molecular mass heterogeneity of the LSU polypeptide in *T. neapolitanus*, even with the addition of a protease inhibitor (13, 21). Recent evidence, based on restriction fragment analyses using *rbcL* gene probes, suggests that multiple forms of the *rbcL* gene may be present in *T. neapolitanus* (7), which could encode multiple protein species. This question remains to be answered.

The results of the immunogold experiments (Fig. 2) indicated that the protein specifically recognized by the anti-TLSU serum is localized in the bacterial symbionts of *S. velum*. All of the symbionts appeared to contain the enzyme since in every section examined all symbionts were labeled. The localization of the gold particles to the cytoplasm indicated that the enzyme exists in *S. velum* symbionts in a soluble form as opposed to inclusion in carboxysomes, RbuP_2Case-containing polyhedral inclusions found in some autotrophic prokaryotes (22). This is consistent with microscopic observations indicating that carboxysomes are not present in *S. velum* symbionts (1, 19).

We conclude from the results of the immunoblot analyses and the immunogold labeling experiments that the intracellular bacterial symbionts are responsible for the RbuP_2Case activity detected in the gill tissues of *S. velum*. The biochemical characterization of RbuP_2Case in *S. velum* remains to be performed, as do more complete metabolic and genetic analyses of the symbionts. It will be interesting to know if any symbiont proteins are encoded in the genome of the invertebrate host as is the case, for example, in most higher plants where the SSU of RbuP_2Case, as well as many other polypeptides, are encoded in the nuclear genome [reviewed by Bogorad (23)].

This study has demonstrated that immunochemical techniques provide an important tool for the characterization of "unculturable" symbionts *in situ*, just as they have been used to localize enzymes in subcellular organelles. The application of these same techniques, using antisera directed against enzymes considered unique to autotrophs or to prokaryotes, will allow more definitive characterization of the autotrophic nature of bacterial symbionts observed in tissues of other marine invertebrates in which RbuP_2Case and other enzyme activities have been detected. By using these methods, it may now be possible to localize such enzymes in symbiotic associations in which (*i*) only low levels of activity are detectable [e.g., the pogonophoran tubeworms (24)] and (*ii*) more than one type of symbiont have been described on the basis of ultrastructure [e.g., in gutless oligochaetes (25)]. Furthermore, these techniques will allow characterization of the symbionts of relatively inaccessible deep-sea hydrothermal vent tubeworms, mussels, and clams, which have thus far eluded culture.

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