Contribution of the Bacterial Endosymbiont to the Biosynthesis of Pyrimidine Nucleotides in the Deep-sea Tube Worm Riftia pachyptila*

The deep-sea tube worm Riftia pachyptila (Vestimentifera) from hydrothermal vents lives in an intimate symbiosis with a sulfur-oxidizing bacterium. That involves specific interactions and obligatory metabolic exchanges between the two organisms. In this work, we analyzed the contribution of the two partners to the biosynthesis of pyrimidine nucleotides through both the "de novo" and "salvage" pathways. The first three enzymes of the de novo pathway, carbamyl-phosphate synthetase, aspartate transcarbamylase, and dihydroorotase, were present only in the trophosome, the symbiont-containing tissue. The study of these enzymes in terms of their catalytic and regulatory properties in both the trophosome and the isolated symbiotic bacteria provided a clear indication of the microbial origin of these enzymes. In contrast, the succeeding enzymes of this de novo pathway, dihydroorotate dehydrogenase and orotate phosphoribosyltransferase, were present in all body parts of the worm. This finding indicates that the animal is fully dependent on the symbiont for the de novo biosynthesis of pyrimidines. In addition, it suggests that the synthesis of pyrimidines in other tissues is possible from the intermediary metabolites provided by the trophosomal tissue and from nucleic acid degradation products since the enzymes of the salvage pathway appear to be present in all tissues of the worm. Analysis of these salvage pathway enzymes in the trophosome strongly suggested that these enzymes belong to the worm. In accordance with this conclusion, none of these enzyme activities was found in the isolated bacteria. The enzymes involved in the production of the precursors of carbamyl phosphate and nitrogen assimilation, glutamine synthetase and nitrate reductase, were also investigated, and it appears that these two enzymes are present in the bacteria.

The environment of deep-sea hydrothermal vents is very dynamic, with rapidly changing physical and chemical parameters. Hot water containing high amounts of dissolved minerals merges from the vent and mixes with the ambient deep-sea water, generating an extreme environment in terms of hydrostatic pressure, high temperature, and chemical toxicity. In addition, this environment totally lacks photosynthesis for animal nutrition; and yet, in these hostile ecosystems, dense communities of microorganisms and animals are found (1, 2).

Riftia pachyptila is a large tube worm found only in the close vicinity of these deep-sea hydrothermal vents in the Pacific Ocean (3). The anatomical organization of R. pachyptila is shown in Fig. 1 (4, 5). The plume is the only part of the worm that comes in free contact with the venting waters; it has a large surface area that is highly vascularized and allows an efficient exchange of metabolites between the environment and the animal. The vestimentum is a muscle that the animal uses to position itself in the tube. Within the large sac formed by the body wall and terminated by the opisthosome are two of the major tissues of the worm: one, the coelomic fluid, bathes the other, the trophosome, which is the symbiont-harboring tissue (3).

R. pachyptila does not possess a digestive tract. The trophosome, which represents 15–30% of the animal mass, is densely colonized by a chemoautotrophic endosymbiotic bacterium (6–8). This tissue is composed of numerous lobules of ~0.15-mm diameter that are vascularized by the circulatory system of the worm. The lobules consist of an outer single layer of cells and the bacteriocytes, the cells containing the bacterial symbiont. The bacteria are typically surrounded by a host vacuolar membrane that includes one or more of these bacteria (3, 9, 10). The trophosome is richly irrigated by small capillaries (2–3-μm diameter), and the maximal diffusion distance from the symbionts to the blood is ~10 μm (3, 6). This organization allows facile metabolic exchanges between the animal and the symbiont (11, 12). The endosymbiotic bacteria and the worm constitute a highly integrated system: the bacteria produce metabolic energy from the oxidation of hydrogen sulfide and provide organic compounds to the worm; in return, the worm provides the bacteria with CO₂, O₂, H₂S, NH₃, and minerals. Thus, this particular nutritional organization involves specific metabolic exchanges between the two organisms.

All living organisms rely on two metabolic pathways for the production of pyrimidine nucleotides. The "de novo" pathway allows the complete synthesis of these nucleotides, including the synthesis of the pyrimidine ring starting with bicarbonate, glutamine, and ATP. The "salvage" pathway ensures the production of these nucleotides from the pyrimidine nucleosides and nucleotide monophosphates provided by the intracellular degradation of nucleic acids.

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* This work was supported in part by CNRS, Université Pierre et Marie Curie, and a grant from the program “DORSALES” of the Institut des Sciences de l’Univers. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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This paper is available on line at http://www.jbc.org
Carbamyl-phosphate synthetase (CPSase), aspartate transcarbamylase (ATCase), and dihydroorotase (DHODase) catalyze the first three steps of the de novo pyrimidine biosynthetic pathway. The organization of these three enzymes differs in different living organisms. In bacteria, all the pyrimidine biosynthetic enzymes are independent proteins encoded by genes generally dispersed throughout the genome, whereas in mammals, the first three steps of this pathway are catalyzed by a single multifunctional protein named CAD (13–15).

The glutamine-dependent CPSase catalyzes the following reaction (Eq. 1).

\[
\text{L-Glutamine} + \text{HCO}_3^- + 2\text{ATP} + \text{H}_2\text{O} \rightarrow \text{NH}_4\text{COOPO}_4^{2-} + 2\text{ADP} + \text{P}_i + \text{L-glutamate (Eq. 1)}
\]

As far as the origin of the CPSase substrates in R. pachyptila is concerned, it has been postulated that carbonic anhydrase plays a significant role in CO₂ uptake by converting CO₂ to HCO₃⁻ (16). The L-glutamine must be synthesized by glutamine synthetase (GSase) from glutamate and ammonia. On the basis of what is known about the general processes of nitrogen assimilation, ammonia may either be obtained from the environment or result from the conversion of nitrate to nitrite by nitrate reductase (NRase) (17) and its reduction to ammonia (18, 19).

The subsequent reactions in the de novo pathway are catalyzed in sequence by ATCase, DHODase, dihydroorotase (DHODase; a membrane-bound or mitochondrial enzyme), orotate phosphoribosyltransferase (OPRTase), and orotidylate decarboxylase, which provides UMP. This nucleotide is then further phosphorylated to UTP, which is aminated into CTP by CTP synthetase (CTPSase) (20). The salvage pathway involves a series of enzymes able to phosphorylate nucleosides and nucleoside monophosphates such as cytidine-uridine kinase and UMP and CMP kinases, enzymes catalyzing the transfer of ribose phosphate such as uracil phosphoribosyltransferase, and enzymes converting one nucleoside into another such as cytidine deaminase (CDase) (20).

Previously reported results showed that CPSase and ATCase, the two first enzymes of the de novo pathway, are present only in the trophosome (21). Here we provide more information about the distribution of the enzymes of the de novo and salvage pathways along with information about the enzymes providing the substrates for CPSase, GSase, and NRase.

### EXPERIMENTAL PROCEDURES

**Chemicals**—[2-14C]cytidine (50.9 mCi/mmol), and [2-14C]uridine (56 mCi/mmol) were purchased from Sigma. l-[14C]aspartate (224.8 mCi/mmol), sodium [14C]bicarbonate (55.0 mCi/mmol), [carboxyl-14C]orotic acid (0.05 mCi/mmol), and [5,6-3H]uridine 5'-triphosphate (14.3 mCi/mmol) were purchased from PerkinElmer Life Sciences. [14C]Uracil (150 mCi/mmol) were purchased from the Commissariat à l’Énergie Atomique (Saclay, France).

**Source and Storage of R. pachyptila Samples**—Samples of the deep-sea tube worm were collected in the East Pacific volcanic range at a depth of 2600 m using the submersible “Nautile” during the campaign HOPE 99. To avoid interference with the subsequent enzyme tests, the specimens were immediately bled and dissected on board, and each isolated organ was frozen in liquid nitrogen as previously described (21).

**Purification of the Bacterial Symbiont**—After collecting and bleeding the animal, the bacterial symbiont was immediately purified by the method proposed by Distel and Felbeck (22) under the conditions previously described (21).

**Protein Extract from Each Organ of R. pachyptila**—Protein extracts from the organs were freshly prepared before the enzyme assays. Frozen tissue (2 g) was suspended in 6 ml of ice-cold extraction buffer (30 mM Tris-HCl, pH 7.8, 10 mM NaCl, 10 mM KCl, 1 mM L-dithiothreitol, 5% (v/v) glycerol, 30% (v/v) ethylene glycol, and 4 µg sodium cacodylate), and the following protease inhibitors were added: 30 µg/ml phenylmethylsulfonyl fluoride, 0.3 mg/ml EDTA, 0.7 µg/ml pepstatin A, and 0.5 µg/ml leupeptin. The mixture was homogenized in a Potter homogenizer with a Teflon pestle. The homogenate was further disrupted by sonication three times for 60 s each with a Biosonic III sonicator at 20 kilocycles/s. The homogenate was then centrifuged at 9000 × g for 20 min, and the resulting supernatant was used for enzyme assays.

**Carbamyl-Phosphate Synthetase Assay**—The activity of CPSase was determined by the radioactive method (23) under the standard conditions described previously by Simon et al. (21).

**Aspartate Transcarbamylase Assay**—The ATCase activity was measured by the radioactive method (24) using l-[14C]aspartate. The standard conditions were 50 mM Tris-HCl, pH 8.0, 1 mM l-[14C]aspartate (specific activity of 0.3 mCi/mmol), and 10 mM carbamyl phosphate. The reaction mixture was incubated at 37 °C for 1 h.

**Dihydroorotase Assay**—The DHODase activity was measured by an assay adapted from Sander et al. (25). The protein extract (50 µl) was incubated for 1 h at 37 °C with 30 mM carbamyl aspartate in 0.1 mM phosphate buffer, pH 6.5, at a final volume of 1 ml. After incubation, 66 µl of 4 M HClO₄ was added to the tubes, which were immersed in an ice bath. The denatured protein was removed by centrifugation. The dihydroorotic acid produced during this incubation was determined after the addition of 1 ml of 1 M NaOH to 1 ml of the extract. The absorbance at 240 nm was recorded, and the data were standardized with reference to authentic dihydroorotate samples.

**Dihydroorotase Dehydrogenase Assay**—The DHODase activity was determined by an adaptation of the method described by Miller (26). The assay mixture was prepared with 50 mM Tris-HCl, pH 8.0, 1 mM
Biosynthesis of Pyrimidine Nucleotides in *R. pachyptila*

All specific enzyme activities were measured at 37 °C. Assays for CPSase, ATCase, DHOase, and OPRTase were performed in 50 mM Tris-HCl, pH 8.0. DHOase activity was determined in 100 mM phosphate buffer, pH 6.5. Assays for CPSase were performed in 50 mM Tris-HCl, pH 8.5. In the case of the isolated bacteria, 100-μl aliquots of extract were used under the same conditions as described for the different *R. pachyptila* tissues, except that the activity of CPSase was determined in 50 mM Britis, pH 7.0. The numbers in parentheses indicate the number of determinations made on different *Riftia* individuals. ND, not detected.

| Body part                  | CPSase(Gln) | ATCase | DHOase | DHOase | OPRTase | CTPSase |
|----------------------------|-------------|--------|--------|--------|---------|---------|
| Branchial plume            | ND          | ND     | ND     | 32 ± 5 (3) | 0.072 ± 0.004 (3) | 0.225 ± 0.026 (3) |
| Vestimentum                | ND          | ND     | ND     | 26 ± 6 (3) | 0.037 ± 0.003 (3) | 0.204 ± 0.012 (3) |
| Trophosome                 | 1.85 ± 0.10 (3)* | 4.2 ± 1.0 (10)* | 0.58 ± 0.19 (3) | 24 ± 4 (3) | 0.147 ± 0.013 (3) | 0.016 ± 0.010 (3) |
| Body wall                  | ND          | ND     | ND     | 53 ± 7 (3) | 0.112 ± 0.009 (3) | 0.124 ± 0.048 (3) |
| Opistosome                 | ND          | ND     | ND     | 20 ± 6 (3) | 0.180 ± 0.015 (3) | 0.196 ± 0.090 (3) |
| Isolated bacteria          | 0.076 ± 0.064 (3) | 2.4 ± 1.4 (4) | 164 ± 40 (3) | 0.072 ± 0.007 (2) | 0.142 ± 0.017 (2) |

* Determined by Simon et al. (21).

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TABLE I

**Distribution of Enzyme Activities of the de Novo Pyrimidine Nucleotide Pathway in Different Parts of *R. pachyptila*** — Since it was previously shown that CPSase and ATCase are present only in the trophosome (21), the distribution of the subsequent enzymes of the pyrimidine pathway in different parts of the worm was examined. The results obtained are presented in Table I. Interestingly, it appeared that the first three enzymes were present only in the trophosome, raising the question of whether these enzymes belong to the bacteria or to the worm. This point was investigated in two different ways.

**Enzyme Activities in the Isolated Symbiotic Bacteria** — The same enzyme determinations were made on extracts from bacteria isolated onboard the ship, immediately after collection of the animals. The results of this analysis are given in Table I. In the bacterial extract, all enzyme activities analyzed were detected, except for glutamine-dependent CPSase (CPSase(Gln)). The instability of CPSases is well known; and most probably, this enzyme was inactivated during the isolation and/or storage of the bacterial preparations. It is noteworthy that partial inactivation of this enzyme was also observed during the analysis of trophosomal extracts by DEAE-Sepharose chromatography.

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phy (see below). The presence of the ATCase and DHOase activities is consistent with the hypothesis supporting the bacterial origin of these enzymes in the trophosome. The OPRTase, DHODase, and CTPSase activities were also present in the isolated bacteria, with the activity of DHODase being particularly high. Thus, it appears that, in contrast to the worm, the bacteria possess all the enzymes of the de novo pyrimidine pathway.

Analyses of Trophosomal CPSase, ATCase, and DHOase Activities by Anion-exchange Chromatography—In eukaryotes, the enzymes that catalyze the first three reactions of the pyrimidine pathway (CPSase(Gln), ATCase, and DHOase) are associated in a multifunctional protein (CAD), whereas in prokaryotes, these three enzymes are separated proteins (39). To determine the origin of these enzymes in the trophosome, extracts were analyzed by DEAE-Sepharose chromatography. The results obtained (Fig. 2) demonstrate that all three activities were distinctly separated. Taken together, these results and those reported above indicate that the first three enzymes of the pathway are of bacterial origin.

Search for Dihydroorotic Acid and Orotic Acid in the Blood of the Worm—The trophosomal location of the three first enzymes and the presence of the subsequent enzymes in all tissues of the worm suggest that the intermediary metabolite dihydroorotate must be delivered to the worm tissues. The high DHODase activity found in the bacteria (Table I) suggests that orotate might also be provided. Consequently, the presence of orotic acid and dihydroorotic acid was examined in the worm blood by colorimetric assays. Orotic acid was found at concentrations of 7.7\(\pm\)1.1 and 12.9\(\pm\)1.9 \(\mu\)M in blood samples from two different animals. Dihydroorotic acid was not detected using the dihydroorotic acid assay, whose limit of detection is \(\approx\)5 \(\mu\)M (38).

Assignment of the Trophosomal Enzymes on the Basis of Their Catalytic Properties—Since the trophosome can contain enzymes from both the bacteria and the worm, we attempted to distinguish between these two possibilities on the basis of some kinetic properties of these enzymes. It has been shown previously that the properties of the trophosomal ATCase (molecular mass, \(K_m\) for aspartate, and sensitivity to allosteric effectors) are characteristic of bacterial enzymes (21). As far as OPRTase and CTPSase are concerned, it can be seen in Fig. 3 (A and B) that their pH activity profiles are significantly different in the trophosome and the vestimentum. The activity of OPRTase in the trophosome was virtually constant from pH 6 to 11. In contrast, OPRTase in the vestimentum showed a clear pH optimum of \(\approx 7.9\) (Fig. 3A). The profiles of the enzymes in the trophosome and the vestimentum are clearly different in the acidic pH range. In an analogous fashion, the CTPSase pH activity profiles in the trophosome and the vestimentum are
very different (Fig. 3B), showing pH optima of 7.2 and 8.7, respectively. These results suggest the existence of different enzyme forms in the trophosome and the vestimentum, suggesting that the trophosome contains a bacterial CTPSase.

On the basis of size-exclusion chromatography, DHOase appears to have a molecular mass of ~110–120 kDa (data not shown), which is comparable to previously determined values in other bacteria (40, 41). The carbamyl aspartate saturation curves shown in Fig. 4 provided a $K_m$ value of 2.5 ± 0.2 mM for this enzyme. Similar results were obtained in the case of Pseudomonas putida DHOase (41). These results are consistent with the bacterial origin of the trophosomal DHOase. Furthermore, the pH dependence of the DHOase activity (Fig. 3C) showed a maximum at pH 6.6, a value that is higher than those found in other bacteria, plants, and mammals (40).

The effect of pH on the activity of CPSase(Gln) is shown in Fig. 3D. The pH optimum determined was 9.6, which is also significantly higher than the known pH optima of homologous enzymes from Escherichia coli (42–44) and mammals (45). It is noteworthy that it was found previously that the ATCase present in the trophosome also exhibits an unusually high pH optimum (21).

**Distribution of the Enzyme Activities of the Salvage Pathways in Different Parts of Riftia**—The results presented above indicate that the worm is unable to synthesize the pyrimidine nucleotides through the de novo pathway. Thus, it must rely on salvage pathways present in the trophosome. For this purpose, the enzymes of salvage pathways were measured in different tissues of the worm. Table II shows the results obtained. NRase activity was detected only in the trophosome, as expected for a bacterial enzyme (46). GSase activity was detected in all tissues tested. The highest activity was found in the plume at the two pH values at which this activity was measured (see below).

To obtain some information about the origin of the GSase activity present in the trophosome, further analysis of this activity was carried out using anion-exchange chromatography. The pH optimum of GSase activity was measured in different tissues of the worm. Table III shows the results obtained. NRase activity was detected only in the trophosome, as expected for a bacterial enzyme (46). GSase activity was detected in all tissues tested. The highest activity was found in the plume at the two pH values at which this activity was measured (see below).

**Enzyme Activities Involved in Nitrogen Assimilation and Synthesis of the Precursors of Carbamyl Phosphate**—The first reaction of the de novo pyrimidine pathway is catalyzed by CPSase using the substrate glutamine, which derives from the process of nitrogen assimilation. To obtain information about the origin of this substrate in *R. pachyptila*, NRase and GSase activities were measured in different tissues of the worm. Table III shows the results obtained. NRase activity was detected only in the trophosome, as expected for a bacterial enzyme (46). GSase activity was detected in all tissues tested. The highest activity was found in the plume at the two pH values at which this activity was measured (see below).

To obtain some information about the origin of the GSase activity present in the trophosome, further analysis of this activity was carried out using anion-exchange chromatography, pH dependence of activity, and thermostability. Two peaks of GSase activity were observed when the trophosomal extract was chromatographed on DEAE-Sepharose, one with high and one with low activity (Fig. 7A). The profile of the trophosomal extract eluted as a single peak that corresponded to the low peak from the symbiont-containing trophosome (Fig. 7B). The major peak of GSase activity present in the trophosome and that in the vestimentum extract were used for the determination of the dependence of their activities on pH. The vestimentum pooled fractions containing GSase activity had a pH optimum of 7.7 (Fig. 8A). This pH optimum and pH activity profile corresponds to that of the vestimentum crude extract (Fig. 8C, not heated). The isolated major peak of GSase in the trophosomal extract has a different pH profile with an optimum of 9.1 (Fig. 8B). The profile of the trophosomal crude extract (Fig. 8D, not heated) appears to be a combination of the pH activity profiles of the main peak of the trophosomal extract and the vestimentum (Fig. 8, A and B).
To further analyze this trophosomal GSase content, the thermostability of these enzymes was investigated. It has been reported that the GSases from prokaryotes are more thermoresistant than those from eukaryotes (47). Consequently, heating is expected to cause inactivation of the GSase of the host, but not of the endosymbiont. To determine the thermal stability of the host and symbiont GSases, the trophosomal and vestimentum protein extracts were heated at 60 °C for 20 min. The

**TABLE III**

| Body part          | NRase activity | GSase activity  |
|--------------------|----------------|-----------------|
|                    | pH 7.7         | pH 9.1          |
|                    | nmol/min/mg protein | nmol/min/mg protein |
| Branchial plume    | ND             | 112.4 ± 0.8 (2) | 93.7 ± 0.4 (2) |
| Vestimentum        | ND             | 43.1 ± 3.8 (2)  | 38.0 ± 1.4 (2) |
| Trophosome         | 1.35 ± 0.04 (2) | 25.7 ± 0.5 (2)  | 26.7 ± 0.3 (2) |
| Body wall          | ND             | 57.5 ± 1.1 (2)  | 50.7 ± 0.6 (2) |
| Opistosome         | ND             | 56.6 ± 0.8 (2)  | 48.6 ± 1.6 (2) |

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enzyme activities measured after the heat treatment of the trophosomal and vestimentum protein extracts and their pH dependences are presented in Fig. 8 (C and D). It can be seen that heating at 60 °C did not inactivate fractions associated with the higher peak from the trophosomal extract (pH optimum of 9.1), but it strongly inactivated both the vestimentum activity and the trophosomal GSase activity centered at pH 7.7. Taken together, these findings indicate that the GSase of the host is an unstable enzyme and explain the low activity of the trophosomal extract. These results indicate that the GSase of the vestimentum activity and in the corresponding fraction of the trophosome. However, the measurements made after separation by chromatography show a significant decrease in the vestimentum activity and in the corresponding fraction of the trophosomal extract. These results indicate that the GSase of the host is an unstable enzyme and explain the low activity of the smaller peak obtained from the trophosomal extract (Fig. 7).

Table III shows that, in the crude extracts, the specific enzyme activity in the vestimentum was higher than that in the trophosome. However, the measurements made after separation by chromatography show a significant decrease in the vestimentum activity and in the corresponding fraction of the trophosomal extract. These results indicate that the GSase of the host is an unstable enzyme and explain the low activity of the smaller peak obtained from the trophosomal extract (Fig. 7).

**DISCUSSION**

In this study, we have investigated in *R. pachyptila* the specific metabolic organization and interdependence between the host and the bacterial symbiont for the biosynthesis of pyrimidines. Our analysis characterized the enzymes involved and their distribution in different tissues of the worm and in the bacteria. Fig. 9 shows the model that summarizes the results of this investigation and that describes the interconnections between the bacteria and its host for the biosynthesis of the pyrimidine nucleotides.

The first three enzymes (CPSase, ATCase, and DHOase) involved in the de novo pyrimidine biosynthetic pathway are present only in the trophosome. Our study has indicated their microbial origin, based on the distribution of enzymes in both the trophosome and the isolated symbiotic bacteria as well as their catalytic and regulatory properties. The apparent molecular mass, *Km*, and sensitivity to effectors of ATCase and CPSase in the trophosome are characteristic of bacterial rather than eukaryotic homologs (21). The complementary results reported here for DHOase (apparent molecular mass and *Km*) also demonstrate the bacterial origin of this enzyme. In addition, all three enzyme activities are found associated with distinct separated proteins, a characteristic of prokaryotic enzymes. In eukaryotes, the first three steps of the de novo pyrimidine nucleotide pathway are catalyzed by a single multifunctional protein, CAD (13–15).

The subsequent enzymes of the de novo pyrimidine pathway (DHOase, OPRTase, and CTPSase) appear to be present in both the bacteria and the worm. The host, *R. pachyptila*, can synthesize pyrimidine from orotate or dihydroorotate provided by the bacteria.

The absence of the de novo pyrimidine biosynthesis has been previously reported for aerobic protozoan parasites such as *Giardia lamblia*, *Trichomonas vaginalis*, and *Trichomonas fetus* (48–50). These parasites appear to rely solely on salvage pathways to obtain their pyrimidine requirements. The results reported in this work show that all tissues of *R. pachyptila* possess the enzyme equipment of salvage pathways for the production of their pyrimidines.

Alternatively, the source of nucleotides or nucleosides for the host might result from release of these products by digestion of the symbiont cells in the trophosomal tissue. Indeed, studies of the trophosome demonstrated the presence of bacterial symbionts in various steps of degradation (51). In this way, these degradation metabolites might be transported and incorporated by the salvage pathways into nucleotides for the host cells. In contrast to nucleotides that are unable to cross cell membranes, nucleosides (uridine, cytidine, uracil, and others) can traverse the membranes (20) and could thereby be delivered to any part of the worm. As far as the bacteria are concerned, the de novo pyrimidine biosynthetic pathway is the only route for the biosynthesis of pyrimidines.

It has not been possible thus far to grow the bacterial symbiont of *R. pachyptila* in culture; and therefore, a thorough investigation has not been completed. However, it appears that the kinetic and regulatory properties of CPSase, ATCase, and DHOase show strong similarity to those of the homologous enzymes from *Pseudomonas* species (21, 46). In addition, the ribosomal RNA sequence comparison of the 5 S and 16 S ribosomes has also showed that the *R. pachyptila* symbiont is closely related to *Pseudomonas* species (52, 53). It is interesting to note that another sequenced protein of the *R. pachyptila* symbiont, the histidine protein kinase, is extremely similar to the homologous enzyme from *Pseudomonas aeruginosa* (54).

The pH dependence studies reported here show that, unexpectedly, CPSase, ATCase, and DHOase as well as GSase of the bacterial symbiont show pH optima for activity that are significantly higher than those of the homologous enzymes from other bacteria. In this regard, it is noteworthy that a comparable observation was made from the genome sequence of the
endocellular bacterial symbiont of the aphid *Buchera* species APS (55). It was shown that the predicted isoelectric points of the products of the open reading frames are, on average, much more basic than those of the homologous proteins from other bacteria. The average pl value of *Buchera* proteins is 9.6, whereas those of *E. coli* and *Hemophilus influenzae* proteins are 7.2 and 7.3, respectively. Thus, the basic character of the proteins might be related to the environment under which the bacterial endosymbionts are living.

The pyrimidine biosynthesis is initiated by the CPSase-catalyzed conversion of HCO$_3^-$, ATP, and L-glutamine into carbamoyl phosphate. The availability of the substrates for this reaction in *R. pachyptila* raises several questions. Carbonic anhydrase, which facilitates the transformation of carbon dioxide into bicarbonate ions, was identified in *R. pachyptila*. This enzyme shows a similarity to enzymes from mammalian sources (17). l-Glutamine plays a key role as a source of nitrogen for the synthesis of nucleotides and is essential for cell proliferation (56, 57). l-Glutamine is synthesized from glutamate and ammonia by GSase, which is a primary enzyme for the assimilation of ammonia. Previous analysis of GSase in *R. pachyptila* showed that GSase is present in the trophosome and the vestimentum (26). However, the existence in the trophosome of only the bacterial enzyme was not entirely established (26). For this reason, we carried out a complementary analysis of GSase activity in other tissues of *R. pachyptila*, and we now have evidence for the existence of distinct forms of GSase in the host and the symbiont. This analysis has shown that all tissues of *R. pachyptila* possess GSase. Furthermore, two forms of GSase were distinguished in the trophosome, one possessing host properties and one showing bacterial properties. Interestingly, the highest GSase activity was found in the branchial plume, strongly suggesting that GSase indeed has an important role in the fixation of ammonia from the vent environment.

Ammonia, a substrate of GSase, may be supplied to this enzyme either from the environment or from nitrate, converted to nitrite by NRase (17), and further reduced to ammonia (18, 19). The NRase activity was previously detected in the trophosomal tissue (18) and in the purified symbiont from *R. pachyptila* (19). The NRase activity was previously detected in the trophosomal tissue (18) and in the purified symbiont from *R. pachyptila* (19). The NRase activity was previously detected in the trophosomal tissue (18) and in the purified symbiont from *R. pachyptila* (19).

The pyrimidine biosynthesis is initiated by the CPSase-catalyzed conversion of HCO$_3^-$, ATP, and L-glutamine into carbamoyl phosphate. The availability of the substrates for this reaction in *R. pachyptila* raises several questions. Carbonic anhydrase, which facilitates the transformation of carbon dioxide into bicarbonate ions, was identified in *R. pachyptila*. This enzyme shows a similarity to enzymes from mammalian sources (17). l-Glutamine plays a key role as a source of nitrogen for the synthesis of nucleotides and is essential for cell proliferation (56, 57). l-Glutamine is synthesized from glutamate and ammonia by GSase, which is a primary enzyme for the assimilation of ammonia. Previous analysis of GSase in *R. pachyptila* showed that GSase is present in the trophosome and the vestimentum (26). However, the existence in the trophosome of only the bacterial enzyme was not entirely established (26). For this reason, we carried out a complementary analysis of GSase activity in other tissues of *R. pachyptila*, and we now have evidence for the existence of distinct forms of GSase in the host and the symbiont. This analysis has shown that all tissues of *R. pachyptila* possess GSase. Furthermore, two forms of GSase were distinguished in the trophosome, one possessing host properties and one showing bacterial properties. Interestingly, the highest GSase activity was found in the branchial plume, strongly suggesting that GSase indeed has an important role in the fixation of ammonia from the vent environment.

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