Membrane-bound proton-translocating pyrophosphatase of *Syntrophus gentianae*, a syntrophically benzoate-degrading fermenting bacterium

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(Received 5 June 1998) – EJB 98 0751/4

Syntrophus gentianae is a strictly anaerobic bacterium which ferments benzoate to acetate, CO_2 and H_2 in the presence of hydrogen-utilizing partner bacteria. Benzoate is activated by a benzoyl CoA ligase enzyme which forms AMP and pyrophosphate as coproducts. Pyrophosphatase activity was found to be largely membrane bound. Pyrophosphate hydrolysis was associated with proton translocation across the cytoplasmic membrane. Proton translocation could be abolished by the protonophor carbonylcyanide *p*-chlorophenylhydrazone, and could also be coupled to ATP formation in membrane vesicle preparations. The ratio of ATP formation/pyrophosphate hydrolysis was 1:3. The reverse reaction, ATP-dependent pyrophosphate synthesis, was possible with the same coupling stoichiometry. Pyrophosphatase was 90% saturated at 1 mM pyrophosphate; pyrophosphate concentrations higher than 5 mM inhibited enzyme activity. Inhibition studies with ATP and EDTA indicated that $MgPP_i^-$ was probably the physiological substrate. The optimum temperature was 35°C. In the presence of Mg^{2+}, the enzyme was remarkably heat stable, with 50% of its maximum activity after 10 min at 60°C. Exogenously added pyrophosphate could not be used for energy conservation.

Keywords: Syntrophus gentianae; pyrophosphatase; proton translocation; ATP synthase; energy conservation.

Pyrophosphate is formed in several reactions for substrate activation by anaerobic bacteria, e.g. in the activation of sulfate by sulfate-reducing bacteria [1] or in the activation of benzoate derivatives during anaerobic degradation of such compounds by nitrate-reducing [2] phototrophic [3] or sulfate-reducing bacteria [4]. In these reactions, pyrophosphate is formed by group transfer from ATP as a side product, and is supposed to be hydrolyzed subsequently by a pyrophosphatase enzyme to shift the overall reaction equilibrium towards product formation [1]. This strategy is efficient but implies a considerable loss of metabolic energy that is released as heat [5].

Alcohols and fatty acids longer than two carbon atoms, benzoate and certain other aromatic compounds are degraded to methane and CO_2 in so-called syntrophic associations of fermenting bacteria with methanogenic partners. The energetical situation of the syntrophic fermenting organisms is comparably difficult; they obtain energy amounts in the range of only fractions of 1 ATP/reaction run in such cooperations [6–8]. These organisms have to save every fraction of an ATP unit, therefore. Assessment of the energetical situation of syntrophically benzoate-degrading bacteria on the basis of substrate, product and hydrogen concentrations in syntrophic cocultures revealed that these organisms have only about 40–45 kJ available/reaction run, equivalent to an overall gain of 1/3–2/3 ATP/substrate degraded [9, 10]. Nonetheless, also the benzoate-degrading *Syntrophus gentianae* activates benzoate by a benzoyl CoA ligase reaction at the expense of 1 ATP, releasing pyrophosphate as coproduct [11]. The pyrophosphatase enzyme present in this organism is characterized in this communication. It is a membrane-bound primary proton pump and represents the first example, to our knowledge, of such an enzyme system in a chemotrophic anaerobic bacterium.

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Abbreviations. CCCP, carbonylcyanide *p*-chlorophenylhydrazone; ACMA, 9-amino-6-chloro-2-methoxyacridine.

Enzymes. ATPase (ATP synthase, proton translocating) (EC 3.6.1.34); pyrophosphatase (EC 3.6.1.1).

MATERIALS AND METHODS

Organisms and cultivation. Cocultures of *S. gentianae* (DSM 8423) with *Methanospirillum hungatei* were taken from our own culture collection. The freshwater mineral medium [12] for cultivation contained 30 mM sodium bicarbonate, 1 mM sodium sulfide as reducing agent, the trace element solution SL 10 [13], selenite-tungstate solution [14], and a seven-vitamin solution [15]. The pH was adjusted to 7.1–7.3. Cultures were grown under N_2/CO_2 (80:20%, by vol.) gas mixture at 28°C. Growth was monitored as change in A_{578}. Cell material for biochemical experiments was grown in 12 l carboys in a fed-batch manner. 4 l were withdrawn every 3–4 days for experiments, and were replaced with complete growth medium. The purity of cultures was checked microscopically during growth in defined mineral medium with benzoate as substrate and after growth tests in complex media containing yeast extract (0.1%), fumarate (5 mM), pyruvate (5 mM) and glucose (5 mM).

Preparation of cell-free extracts. Cell suspensions of the coculture of *S. gentianae* with *M. hungatei* were harvested to the end of the exponential growth phase, transferred into oxy-

gen-free centrifuge vials in an anoxic chamber (Coy) and centrifuged at 8000×*g* for 30 min. Cells were resuspended in mineral medium without benzoate and centrifuged again. For selective cell lysis, the pellet was resuspended in Tris/HCl (10 mM, pH 8.0) containing 0.5 mg/ml lysozyme and 10 mM EDTA. The final concentration of lysozyme was 2.5–5 μg/mg cell protein. Cells were incubated for 30–45 min at 37 °C, and lysis was checked for microscopically.

For localization experiments (association with cell membranes or cytoplasm), the buffer contained in addition to lysozyme and EDTA 20% sucrose (mass/vol.). Spheroplasts were centrifuged and lysis was initiated later by addition of $MgCl_2$ to a final concentration of 10 mM.

Fractionation of cell-free extracts. 3 ml sucrose solution (20% mass/vol.) was overlayed with 0.5 ml cell-free extract containing less than 10 mg protein/ml. This set was centrifuged for 90 min at 100000×*g* and 4 °C in an Optima TL-Ultracentrifuge (Beckman Instruments). The supernatant (cytosol) was carefully removed in an anoxic chamber. The membrane containing pellet was diluted in Tris/HCl, pH 8.0, washed once, centrifuged again under the same conditions, and resuspended in the same buffer.

Determination of enzyme activities. Enzyme activities were measured either continuously by fluorescence spectroscopy or discontinously by quantification of product formation and substrate utilization. All activities were measured at room temperature if not stated otherwise, in at least three parallel experiments. Proportionality of the enzyme activity with the protein content was checked in every case. Controls were also run with heat-denatured extracts to ensure that the activities found were catalyzed by enzymes.

Enzyme assays. Inorganic pyrophosphatase was measured following the formation of inorganic phosphate by the method of Jetten et al. [16]. In addition, the pyrophosphate concentration decrease was measured by application of a commercially available enzyme kit (Sigma Chemical Co.). In this test system, pyrophosphate is cleaved by a pyrophosphate-dependent fructose-6-phosphate kinase and coupled via aldolase, triosephosphate isomerase and glycerol phosphate dehydrogenase to oxidation of NADH which was measured at 340 nm.

Protein content. Protein concentrations were determined by a commercially available kit (Pierce) essentially after the method of Bradford [17]. Bovine serum albumin served as a standard.

Assays for proton translocation. Fluorescence spectrophotometric assays of proton translocation were carried out after addition of 2–8 μM 9-amino-6-chloro-2-methoxyacridine (ACMA) to membrane vesicle preparations. Excitation wavelength was 410 nm, and emission was measured at 490 nm in a Perkin Elmer Luminescence Spectrometer LS 50 equipped with a stirred optical cell.

Inhibition experiments. Carbonylcyanide chlorophenylhydrazone (CCCP) was added from ethanolic stock solutions to a final concentration of 2 nmol/mg protein. Controls were run by adding equal volumes of pure ethanol.

Chemical analyses. ATP, ADP and AMP were separated and measured via ion pair chromatography [18]. The stationary phase was Ultrasphere ODS (5 μ diameter in a 4.6×150 mm column, Beckman Instruments), mobile phase 80 mM KH_2PO_4 (adjusted with 1 M KOH to pH 6.0), 5 mM tetrabutylammonium hydrogensulfate in methanol (77:23, by vol.) at a flow rate of 1.25 ml/min. Detection was at 260 nm.

RESULTS

Localization of pyrophosphatase activity. Since *S. gentianae* has to save energy far more than other microorganisms, we checked whether this bacterium is able to conserve energy from pyrophosphate cleavage. Localization experiments revealed that the pyrophosphatase activity was largely membrane-bound, provided that a gentle procedure for cell breakage was applied (Table 1). 3-Hydroxybutyryl-CoA dehydrogenase was taken as a marker enzyme of the cytoplasmic fraction and was detected in the membrane fraction only at negligible activities, indicating that the membrane fraction was essentially free of cytosolic enzymes. By addition of Triton X-100 to cell-free extracts, pyrophosphatase could be resolved from the membrane.

Table 1. Localization of enzyme activities in the cytosolic and the membrane fraction of *S. gentianae*. Pyrophosphatase +1% Triton X-100; activity after addition of 1% Triton X-100 to the cell-free extract.

Enzyme	Relative activity in		
	cell-free extract	membrane fraction	cytoplasmic fraction
	%		
3-OH-Butyryl CoA dehydrogenase	100	2.5	105.4
Pyrophosphatase	100	63.3	17.3
Pyrophosphatase + 1% Triton X-100	100	0	78.4

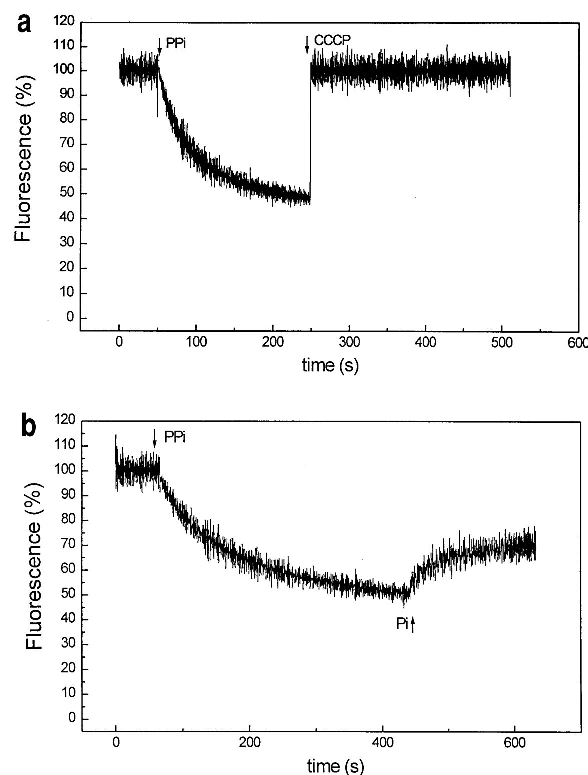

Fig. 1. Quenching of ACMA fluorescence in ACMA-loaded membrane vesicle preparations of *S. gentianae*. (a) Effect of 1 mM pyrophosphate addition and subsequent addition of 2 μM CCCP. (b) Effect of 0.1 mM pyrophosphate addition and subsequent addition of 2 mM phosphate.

Proton translocation across the cytoplasmic membrane by pyrophosphate cleavage. Inside-out cytoplasmic membrane vesicles were prepared by application of the lysozyme/EDTA

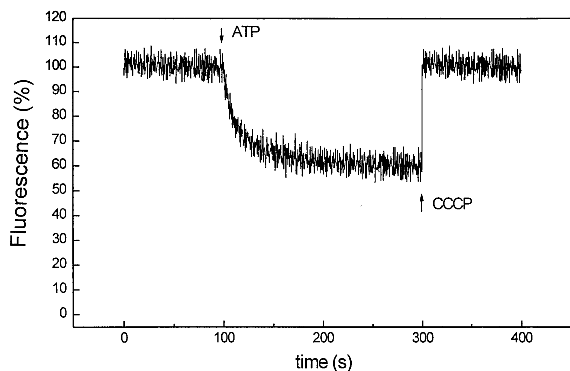

Fig. 2. Quenching of ACMA fluorescence in ACMA-loaded membrane vesicle preparations of *S. gentianae*. Effect of 1 mM ATP addition and subsequent addition of 2 μM CCCP.

technique as described. For analysis of a possible proton-translocating activity of the pyrophosphatase, the proton-sensitive fluorescent dye ACMA was applied. Protonation of ACMA results in quenching of its fluorescence which allows a very sensitive optical measurement of proton translocation into ACMA-loaded vesicles. Release of protons from vesicles by the protonophor CCCP results in an increase of ACMA fluorescence. Addition of 1 mM pyrophosphate from a solution of exactly the same pH as the vesicle suspension to ACMA-loaded vesicles resulted in a quenching of fluorescence (Fig. 1 a), indicating that protons were translocated into the vesicles. Addition of CCCP alleviated this quenching effect. The effect could be partly alleviated if only 0.1 mM pyrophosphate was applied. After proton accumulation due to pyrophosphate cleavage, addition of 2 mM phosphate caused an increase in fluorescence (Fig. 1b) indicating a decrease of the proton gradient across the membrane due to partial inhibition of the pyrophosphatase reaction by high levels of its reaction product phosphate. This effect was not observed in the presence of 1 mM pyrophosphate, in accordance with an assumed product inhibition of the pyrophosphatase reaction.

Proton translocation by ATPase activity. Since a membrane-bound proton-pumping ATPase might use the proton gradient established by the pyrophosphatase reaction for ATP formation, we checked for this enzyme activity as well. Addition of ATP to ACMA-loaded vesicle preparations caused a strong quenching of fluorescence that could be neutralized by addition of CCCP (Fig. 2).

Coupling of pyrophosphatase with ATP synthase activity. If the proton accumulation caused by pyrophosphate hydrolysis can be used for ATP formation, this should be easy to follow in membrane vesicle preparations. Addition of pyrophosphate to membrane vesicles loaded with ACMA caused a quenching of fluorescence which was partially neutralized upon addition of ADP, indicating a proton gradient-dependent formation of ATP by the membrane bound ATPase (Fig. 3 a). Thus, it was possible to couple ATP formation to pyrophosphate cleavage. Nevertheless, deprotonation of ACMA was incomplete since the final fluorescence was about 10% less than the initial fluorescence. Control experiments carried out without addition of vesicles showed no effect at all on the total fluorescence.

Also, the reverse reaction could be demonstrated (Fig. 3 b). If ATP was added to membrane vesicles loaded with ACMA, the fluorescence was quenched indicating a proton translocation into the vesicles. Addition of phosphate partially neutralized this effect indicating an efflux of protons out of the vesicles as a consequence of pyrophosphate formation. Addition of phosphate

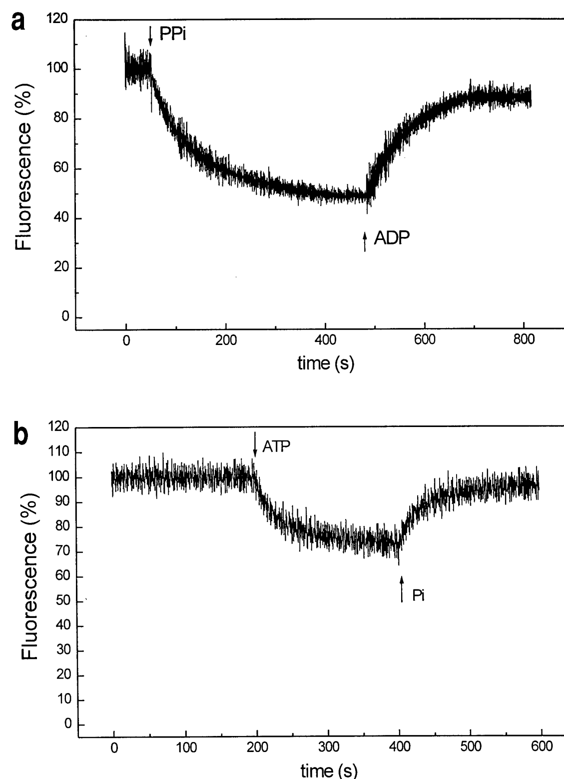

Fig. 3. Quenching of ACMA fluorescence in ACMA-loaded membrane vesicle preparations of *S. gentianae*. (a) Effect of 1 mM pyrophosphate addition and subsequent addition of 1 mM ADP. (b) Effect of 1 mM ATP addition and subsequent addition of 2 mM phosphate.

or ADP plus phosphate to vesicle preparations had no effect on the fluorescence.

Control experiments in non-continuous enzyme assays comfirmed that CCCP had no effect on ATPase or pyrophosphatase activity.

Stoichiometry of pyrophosphatase versus ATPase activity in vesicle preparations. The ratio of pyrophosphate cleavage to ATP synthesis by membrane vesicle preparations was measured discontinuously in time-dependent experiments. The ratio of cleaved pyrophosphate/ATP formed was approximately 3.7 : 1 (Fig. 4 a). This value varied with preparations between 3.1 : 1 and 4.5 : 1. For measurement of the backwards reaction, ATP and phosphate were added to vesicles and cleavage of ATP was followed simulaneously with formation of ADP and pyrophosphate (Fig. 4 b). The ratio of ATP cleaved/ pyrophosphate formed was approximately 1 : 3.3. Also, this value varied with vesicle preparations between 1 : 2.4 and 1 : 3.5. Therefore, reactions were measured in both directions with the same vesicle preparations (0.05 mg · ml^{-1} protein content). Table 2 lists the specific activities of both enzymes in the coupled assays for both reaction directions.

Further characterization of pyrophosphatase activity of *S. gentianae*. Saturation of pyrophosphatase with pyrophosphate revealed that the enzyme was saturated at 2 mM pyrophosphate. 90% of the maximum reaction rate was reached with 1 mM pyrophosphate. Pyrophosphate concentrations of 5 mM and higher inhibited pyrophosphatase activity (results not shown).

The enzyme exhibited an activity maximum at 35°C. At 20°C and 50°C, the activity was about 50% of the maximum. For determination of the heat stability of enzyme activity, the

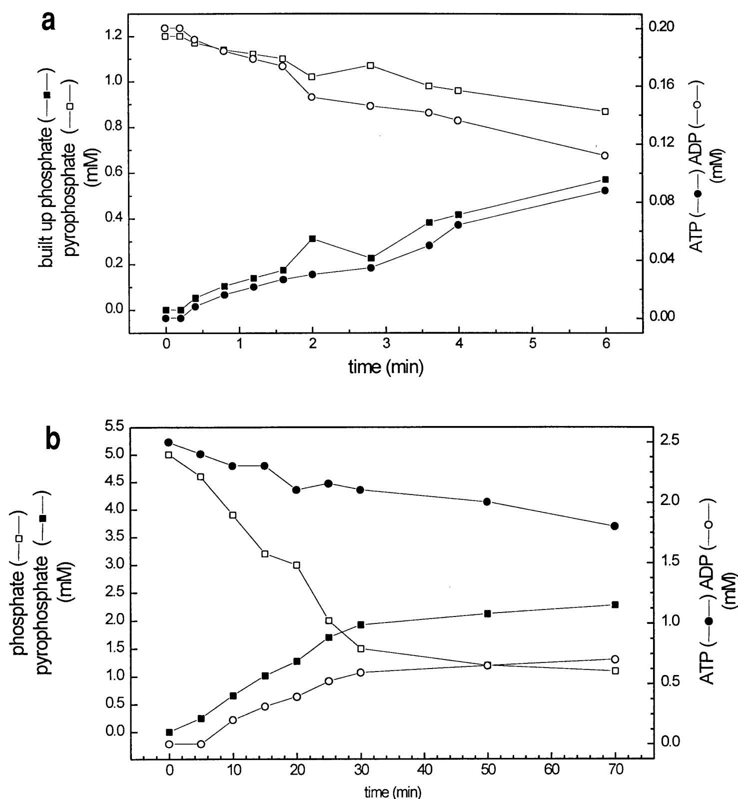

Fig. 4. Coupling of pyrophosphate hydrolysis with ATP synthesis and vice versa in membrane vesicle preparations of *S. gentianae*. (a) Transformation of pyrophosphate and ADP to ATP and P_i. (b) Transformation of ATP and P_i to ADP and pyrophosphate by the same membrane vesicle preparation.

Table 2. Specific reaction activities of enzymes in experiments shown in Fig. 4 a, b.

Direction of reaction	Pyrophosphatase	ATPase
	μmol · min^{-1} · mg protein^{-1}	
ATP cleavage/pyrophosphate formation	0.65	0.2
Pyrophosphate cleavage/ATP formation	1.1	0.3

enzyme was exposed to various temperatures for 10 min and the activity was measured afterwards at 35 °C. The enzyme was stable up to 60 °C without activity loss; incubation at temperatures above 60 °C caused a significant loss of at least 50% activity. Low temperatures did not impair the enzyme (results not shown).

Prophosphatase activity was strongly inhibited by ATP and EDTA (Table 3). In both cases, inhibition could be reverted by addition of 10 mM Mg^{2+}. Bubbling air through the extracts caused a decrease of activity of about 15% but this effect was probably due to mechanical denaturation rather than to oxygen inactivation because bubbling with nitrogen had the same effect.

Determination of growth yields in the presence and absence of exogenously provided pyrophosphate. In order to check whether exogenously provided pyrophosphate could be used as an energy source, cultures of *S. gentianae* were supplemented with 5 mM pyrophosphate in addition to benzoate. Controls were run with benzoate as sole substrate. Since addition of pyrophosphate caused strong formation of turbidity, additional controls were run with benzoate plus pyrophosphate free of inoculum (sterile). After four weeks of incubation, the values of A_{578} after correction for the background turbidity gave no indication of a significant growth yield increase by pyrophosphate utilization. Also, the protein content and the amount of benzoate degraded in both cultures were identical. Moreover, the pyrophosphate provided had not been hydrolyzed.

Table 3. Inhibition of membrane-bound pyrophosphatase activity of *S. gentianae*.

Inhibitor (5 mM)	Remnant activity
	%
None	100
ACMA (5-μM)	98
Titanium (III) citrate	87
NaN_3	76
Sodium dithionite	74
KCN	65
EDTA	36
EDTA + 10 mM $MgCl_2$	88
ATP	33
ATP + 10 mM $MgCl_2$	85

DISCUSSION

In the present communication, a membrane-bound pyrophosphatase enzyme from a syntrophically benzoate-fermenting bacterium is described which acts as a primary proton pump and conserves metabolic energy through the establishment of a proton gradient across the cytoplasmic membrane. Coupling experi-

ments indicated that about 1/3 of an ATP unit is conserved/ pyrophosphate hydrolysis reaction.

The pyrophosphatase enzyme of *S. gentianae* is Mg^{2+}-dependent, similar to the pyrophosphatases of *Escherichia coli* and *Saccharomyces cerevisiae* [19]. The relationship between Mg^{2+} concentrations and concentrations of pyrophosphate has been described in detail for *E. coli* and *S. cerevisiae* [19–22]. It was suggested that $MgPP_i^-$ is the actual substrate of this reaction [19, 20]. As described also for the enzymes from *E. coli*, *S. cerevisiae* and *Methanosaeta soehngenii*, these pyrophosphatase activities are inhibited by pyrophosphate at higher concentrations [16, 19]. Similar to the pyrophosphatases of other Gram-negative bacteria [19, 23, 24], also the *S. gentianye* enzyme was remarkably heat-stable in the presence of magnesium ions.

Membrane-bound pyrophosphatases have been described earlier to be present in the anaerobic phototrophic bacterium *Rhodospirillum rubrum* [25–28] and some other anoxygenic phototrophs [29], as well as in eukaryotic cells, e.g. plant tonoplast membranes [30] and mitochondria [31]. It was assumed that this enzyme in anoxygenic phototrophs helps to conserve metabolic energy under conditions of energy deprivation [32, 33]. Since the proton-pumping pyrophosphatase activity turned out to be reversible, its physiological function could be light-induced maintenance of a certain pyrophosphate level in growing cells as suggested from experiments with *Rhodopseudomonas viridis and Rhodobacter capsulatus* [28, 34]. Also, the pyrophosphatase found in the acetate-cleaving methanogen *Methanosaeta* (*Methanothrix*) *soehngenii* appeared to be membrane-associated, but this enzyme was only weakly bound to the membrane and possible implications in energy metabolism could not be resolved [16].

Measurement of proton translocation with the proton-sensitive fluorochrome ACMA in the presence and absence of the protonophore CCCP in isolated membrane vesicles of *S. gentianae* indicated that this enzyme acts as a primary proton pump in a reversible manner. Coupling experiments with the ATPase present revealed that these two enzymes in concert can reversibly synthesize ATP upon pyrophosphate hydrolysis and vice versa. The stoichiometry of ATP hydrolysis to pyrophosphate synthesis was 1:3, and 3:1 for the reverse reaction. Thus, the coupling efficiency was unusually good. The coupling stoichiometry indicates that pyrophosphate hydrolysis translocates one proton across the cytoplasmic membrane if the ATPase translocates three protons/reaction run, analogous to ATPases of other bacteria [35]. The standard free energy change of pyrophosphate hydrolysis is −22 kJ/mol [5]. In the growing cell (pyrophosphate assumed to be 1 mM, phosphate 10 mM) [5], the free energy change is not significantly different (−28 kJ/mol); under the reaction conditions in our experiments (pyrophosphate and phosphate in the range of 1 mM) it was about −40 kJ/mol. The free energy bound in reversible ATP formation from ADP and P_i under physiological conditions is about 50 kJ mol^{-1} (Thauer et al., 1977). Depending on the pyrophosphate concentration in the cell, a translocation of one or two protons across the membrane appears to be possible, leading to a coupling ratio between pyrophosphate hydrolysis and ATP synthesis of 1:3 to 2:3. Thus, the experimentally observed coupling stoichiometry of pyrophosphatase and ATPase in our membrane vesicle preparations matches rather well the thermodynamically possible energy transfer between both reactions.

In the overall energy balance of benzoate degradation by *S. gentianae*, synthesis of 1/3 ATP by pyrophosphate hydrolysis constitutes an essential fraction of the overall energy balance of this organism [11]. Other unusual steps in energy metabolism of this organism are a membrane-bound, sodium-ion-translocating glutaconyl-CoA decarboxylase and a new, very energy-efficient benzoyl-CoA-dearomatizing reductase [11]. Thus, energy conservation by pyrophosphate hydrolysis is an essential part in the energy balance of this bacterium, different from the situation of the phototrophic bacteria mentioned above where this reaction may contribute to the overall energy budget only to a minor degree.

Despite the obvious energy conservation in pyrophosphatase hydrolysis, we could not observe growth of this organism with pyrophosphate, or an increase in growth yields in the presence of exogenously provided pyrophosphate. Nonetheless, this does not disprove our finding of energy conservation by hydrolysis of endogenous pyrophosphate; obviously, *S. gentianae* has no uptake system for exogenous pyrophosphate. Microbial growth by pyrophosphate hydrolysis has been reported repeatedly in the past [36–38] but these claims have never been evaluated on a quantitative or biochemical basis, and could not be repeated by other laboratories. The reported turbidity increase by pyrophosphate addition were probably due to precipitation of ill-soluble pyrophosphate salts as we observed this to happen also in our sterile control assays.

The authors appreciate helpful suggestions by Drs Peter Dimroth, Wolfgang Buckel, and Mike Jetten. This research was supported by a grant of the *Deutsche Forschungsgemeinschaft*, Bonn-Bad Godesberg.

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