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Characterizing the Function of Amino Acid Transporter ACYPI008971 in the Pea Aphid/Buchnera Symbiosis

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CHARACTERIZING THE FUNCTION OF AMINO ACID TRANSPORTER
ACYPI008971 IN THE PEA APHID/*BUCHNERA* SYMBIOSIS

By

Yu-Ching Hsu

A THESIS

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Master of Science

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Yu-Ching Hsu

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Characterizing the Function of Amino Acid
Transporter ACYPI008971 in the Pea Aphid/*Buchnera* Symbiosis

Abstract of a thesis at the University of Miami.

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Sap-feeding insects have associated with bacterial symbionts for hundreds of millions of years. These symbioses have increased insect fitness, facilitating their persistence through evolutionary time. The symbionts of sap-feeding insects provide their hosts with amino acids that are found at very low concentrations in their diets. Typically amino acid metabolism is partitioned between host and symbiont so that the host synthesizes non-essential amino acids while the symbiont contributes essential amino acids. The biosynthesis of some amino acids however, requires enzymes encoded by both the host and the symbiont. Understanding how amino acid metabolism is integrated between insect host and bacterial symbiont remains unclear. Functional characterization of transporters that exchange amino acids between host and symbiont can advance understanding of host/symbiont metabolic integration. In this thesis I used the pea aphid/*Buchnera* symbiosis model to advance understanding of host/symbiont metabolic integration.

Here I report on the functional characterization of *Acyrtosiphon pisum* amino acid transporter ACYPI008971. Using two-electrode voltage clamp electrophysiology (TEVC) I show that ACYPI008971 responds to proline, alanine and

glycine, leading me to speculate that ACYPI008971 is a transporter of these three amino acids.

Based on the response of ACYPI008971 to proline, alanine and glycine I present an advanced model of host/symbiont integration of amino acid metabolism in the *A. pisum/Buchnera* symbiosis.

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Chapter1: Introduction

Symbiosis

The German mycologist, Heinrich Anton de Bary (1879), discovered symbiosis, defining it as “unlike organisms living together”. The “unlike organisms” are now known as host and symbiont. Many kinds of organisms are “living together” in symbiotic relationships thereby increasing their fitness and persisting through evolutionary time.

Based on the relationship between host and symbiont, there are three different types of symbiosis; parasitism, commensalism and mutualism. Parasitism shows an unbalanced relationship between the parasite and its host. Parasites always gain benefits but cause harm to the host; for example the human head louse, a human ectoparasite that feeds on human blood, causes extreme itching of the human head. Commensalism, also an unbalanced relationship, occurs when the symbiont obtains benefits from the host but the host gains neither benefit nor harm. Mutualism, on the other hand, is a well-balanced relationship in which both partners in the team gain benefits from each other, for example, phloem sap-feeding insects and their symbiotic bacteria. The model system of this thesis, that of the pea aphid, *Acyrtosiphon pisum*, and its obligate, intracellular bacterial symbiont, *Buchnera aphidicola*, is a mutualism.

Cellular arrangement of the A. pisum/Buchnera symbiosis

Phloem sap, the sole diet of aphids, is nutritionally poor and unbalanced. Thus, aphids like other phloem sap-feedings insects rely on their bacteriocyte (also called mycetocyte) endosymbionts for provision of essential nutrients, including amino acids for growth

and reproduction (Douglas, 1998). Aphid primary endosymbionts are housed inside the specialized insect cells called bacteriocytes and therefore nutrient exchange between host and symbiont is critical for the function of this mutualism. The *A. pisum/Buchnera* symbiosis is the most extensively studied insect model for identifying the nutrient interaction between host and symbiont (Moran et al, 2008; Moran and Degnan, 2006 and Elsik, 2010). Studies on the ultrastructure of the bacteriocyte show that each individual *Buchnera*, with its inner and outer gram-negative bacterial membranes, is surrounded by an aphid-derived envelope called the symbiosomal membrane, with thousands of *Buchnera* housed in each bacteriocyte cell (Houk and Griffiths, 1980; Houk et al, 1977; Melean and Houk, 1973) (Figure, 1.2). Understanding the cellular structure of bacteriocytes informs study of collaborative amino acid metabolism in the aphid/*Buchnera* symbiosis, where it is inferred that non-essential amino acids synthesized by the aphid will be transported from aphid to *Buchnera* and essential amino acids made by *Buchnera* will be transported in the opposite direction from *Buchnera* to aphid (Macdonald et al, 2012). While the metabolic contributions of host and symbiont can be inferred from their genomes, the mechanisms that integrate metabolism between host and symbiont are largely unknown.

Glutamine transporter ApGLNT1 regulates amino acid biosynthesis in the A. pisum/Buchnera symbiosis

The *A. pisum* (International Aphid Genomic Consortium, 2010) and *Buchnera* (Shigenobu et al, 2000) genomes have both been sequenced. Shigenobu et al (2000) found that *Buchnera* had lost most non-essential amino acid biosynthesis genes such as the enzymes for serine biosynthesis. The question of how *Buchnera* obtains the non-

essential amino acids it is unable to synthesize remains open. Aphids synthesize some non-essential amino acids by taking precursor amino acids from their phloem sap food, and following synthesis, provide these non-essential amino acids to *Buchnera*. Phloem sap contains high amounts of asparagine and aspartate (Douglas, 2006). Shigenobu and Wilson (2011) indicated that aphids mediated the conversion of asparagine and aspartate and transamination of aspartate to oxaloacetate while releasing glutamate at the same time (Figure 1.3). On the other hand, aphids also mediate the conversion of glutamate and glutamine in the bacteriocyte cytoplasm (Shigenobu and Wilson, 2011) (Figure 1.3). *A. pisum* can synthesize glutamine (Sasaki and Ishikawa, 1993) and aphid hemolymph contains high levels of glutamine and asparagine (Sasaki and Ishikawa, 1995). Previous studies on *A. pisum* nitrogen metabolism showed that compared with aposymbiotic aphids (antibiotic treated aphids cured of their *Buchnera* symbionts), the glutamine level in symbiotic aphids decreased significantly between 42 and 48 days of age (Sasaki, 1990). Using ^{15}N labeled glutamate Sasaki and Ishikawa (1995) found that glutamine is transported from aphid hemolymph across the cell membrane of bacteriocytes into the cytosol. A recent functional study identified an *A. pisum* glutamine transporter, ApGLNT1 (ACYPI001018), that is highly expressed in bacteriocytes compared to the house-keeping gene GAPDH (Price et al, 2014). ApGLNT1 localizes to the cell membrane of bacteriocytes and transports glutamine from the host hemolymph into bacteriocytes (Price et al, 2014). Arginine, synthesized by *Buchnera*, generates a feedback inhibition on ApGLNT1 (Price et al, 2014). Price et al (2014) argue that essential amino acid biosynthesis by *Buchnera* is shut-down when arginine accumulates in aphid hemolymph by inhibiting glutamine transport by

ApGLNT1. Price et al's model of arginine feedback inhibition of ApGLNT1 is consistent with the previous proposal that host aphids regulate amino acid biosynthesis in bacteriocytes by controlling supply of non-essential amino acids to *Buchnera* (Moran et al, 2003 and Thomas et al, 2009).

There are three different types of membrane in bacteriocytes that are relevant to regulation of amino acid production, they include the bacteriocyte cell membrane, the symbiosomal membrane and the *Buchnera* inner and outer membranes. Price et al (2014) found that glutamine transporter ApGLNT1 localized to the bacteriocyte cell membrane, in contrast, the transporters located on symbiosomal membrane and *Buchnera* inner and outer membranes have not yet been identified. The goal of this thesis is to functionally characterize *A. pisum* amino acid transporter ACYPI008971, a transporter that localizes around individual *Buchnera* cells (Feng, unpublished data). This work has the potential to advance understanding of host/symbiont integration of amino acid metabolic pathways between aphid and *Buchnera*.

Eukaryotic-specific amino acid/auxin permease family (AAAP family) amino acid transporters

Over twenty families or superfamilies of membrane transporters have been identified (Paulsen et al, 1998; Saier, 2000). Each transporter family has been classified by their DNA sequence similarity and their substrate specificity (Saier and Reizer, 1991; Saier 1994, 1996, 1998; Paulsen et al, 1998). The eukaryotic-specific amino acid/auxin permease (AAAP) family is a eukaryotic amino acid transporter family (Saier et al, 2000) that commonly transports auxin and single or multiple amino acids across membranes by symport (Saier, 2000, Jack et al, 2000).

Two-electrode voltage clamp electrophysiology (TEVC)

To test the function of membrane protein transportation, two-electrode voltage clamp electrophysiology (TEVC) has been introduced. The technique of voltage clamp was inspired by two milestones of modern electrophysiology, which are Hodgkin-Huxley equation by A. L. Hodgkin and A. F. Huxley (1952) and single-channel events by E. Neher and B. Sakmann (1976). TEVC has now become the most powerful way for functional characterizing membrane proteins.

The basic idea of TEVC is to keep the membrane potential constant by injecting current equal and opposite to the currents produced by the cell (Guan et al, 2013). Many types of amino acid transporters co-transport amino acids and ions; therefore in a *Xenopus* oocyte expressing an amino acid transporter, amino acid specificity can be tested by presenting different amino acids to a voltage clamped oocyte and testing which amino acids are associated with ionic currents.

Limitations of TEVC

Although TEVC is the most powerful way to test the function of membrane protein, the limitation of the technique is that it measures ion flux as a proxy for amino acid transport. As a complimentary assay to TEVC, isotope uptake assays can be used to directly measure the uptake of the labeled amino acid. An additional limitation of the TEVC technique is that it can only detect transport by ion-coupled transporters since the measurement is based on the movement of those ions.

Research aims

In this thesis I investigate the function of *A. pisum* amino acid transporter

ACYPI008971 using TEVC electrophysiology. I injected synthetic ACYPI008971 RNA into *Xenopus laevis* oocytes. After the oocytes have been through three days of incubation to allow expression of the exogenous RNA, I tested transport response using an automated parallel electrophysiology system (OpusExpress 6000A, Molecular Devices). Six different concentrations of proline, alanine and glycine were applied as substrates for ACYPI008971 response with leucine included as a negative control. My results are TEVC traces showing ACYPI008971 response to different concentration of proline, alanine, glycine and leucine. I used pClamp software to measure the amplitude of each trace. The quantitative data generated from electrophysiology current traces facilitated Michaelis-Mentens kinetic analysis using Prism software. Finally, I interpret my results to advance understanding of host/symbiont metabolic integration in the *A. pisum*/*Buchnera* symbiosis.

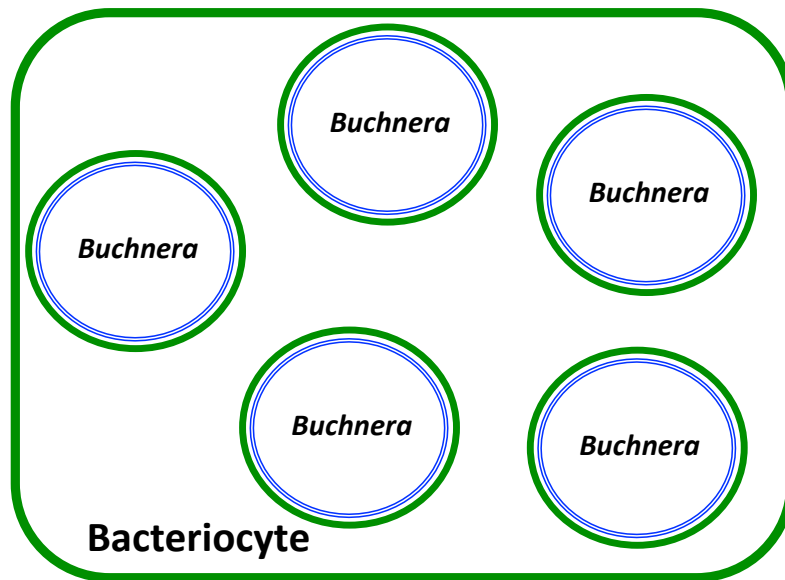
Chapter 1 Figures

Figure 1.1 Three types of membranes of bacteriocyte. The rectangular green line represents the aphid-derived bacteriocyte membrane. Each circle is a single *Buchnera*. Thousands of *Buchnera* are housed in a bacteriocyte. The circular green line indicates the aphid-derived symbiosomal membrane. The double blue lines represent the *Buchnera* inner and outer membranes.

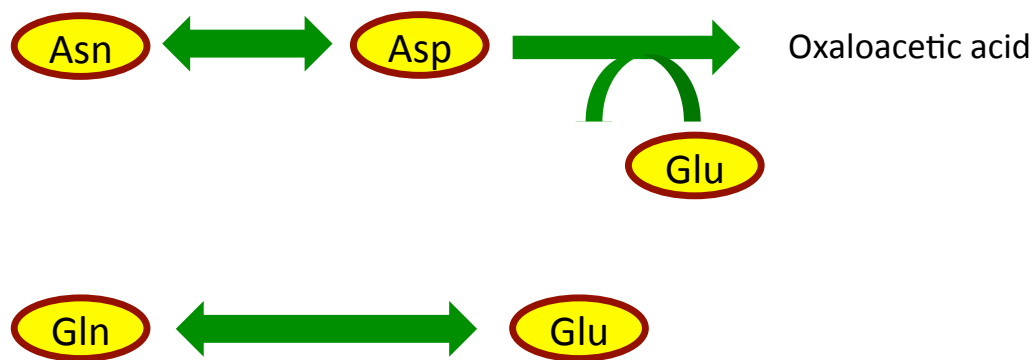


Figure 1.2 Amino acid metabolic pathways. Modified from Shigenobu and Wilson (2011). Asn: asparagine; Asp: aspartate; Gln: glutamine and Glu: glutamate. Arrows represent the direction of amino acid biosynthesis mediated by aphid. Glutamate is the by-product of oxaloacetic acid biosynthesis.

Chapter 2: *Acyrtosiphon pisum* amino acid transporter ACYPI008971 responds to proline, alanine and glycine.

Summary

The goal of this thesis was to identify the substrates that can be transported by *A. pisum* amino acid transporter ACYPI008971. Previous research on *Drosophila melanogaster* (CG1139) and mammalian (SLC36) orthologs of ACYPI008971 demonstrated that they transport proline, alanine and glycine at pH5.5 (Goberdhan et al. 2005, Thwaites et al, 2011; Kennedy et al, 2005). Thus, I hypothesized that ACYPI008971 would also transport proline, alanine and glycine at pH5.5. In order to test this hypothesis, I microinjected ACYPI008971 (LOC100168251) complementary RNA (cRNA) into *Xenopus laevis* oocytes and used two-electrode voltage clamp electrophysiology to measure currents generated by ACYPI008971 in response to the addition of different amino acid substrates. I found that ACYPI008971 has some responses to 2mM proline, alanine and glycine at pH5.5, but that oocyte survival was poor. The bad quality of RNA is a possible cause of low oocyte survival rate. Therefore, I re-synthesized the cRNA. I performed a dose dependent experiment at pH 7.4 using the newly synthesized cRNA. Six different concentrations of proline, alanine and glycine were tested. The results of the dose-dependent experiment showed that ACYPI008971 produces currents in the presence of proline, alanine and glycine and that the magnitude of the response increases with increasing substrate concentration. Based on my data, ACYPI008971 can transport proline, alanine and glycine at pH7.4.

Background

A previous study on the gene expression of 17 *A. pisum* amino acid transporters showed that five (ACYPI000536, ACYPI000550, ACYPI001018, ACYPI008904 and ACYPI008971) of those 17 genes were expressed in bacteriome at the level of more than 10% of GAPDH expression (Price et al, 2014). Functional characterization of all five transporters identified ACYPI001018 (ApGLNT1) as a strong glutamine transporter that transported 7.6 fold more glutamine than ACYPI008904, which also significantly transported glutamine relative to the control (Price et al, 2014). The substrates of the other three transporters (ACYPI000536, ACYPI000550 and ACYPI008971) have not yet been identified. In 2013, my lab mate Honglin Feng, using a custom monospecific anti-ACYPI008971 antibody, showed that ACYPI008971 localizes to the symbiosomal membrane, the host-derived membrane that surrounds individual *Buchnera* cells (Shigenobu and Wilson, 2011). The goal of this thesis is to determine the amino acid substrate(s) of ACPYI008971.

ACYPI008971 belongs to the eukaryotic-specific amino acid/auxin permease (AAAP) family of amino acid transporters (Price et al, 2011). A phylogenetic analysis of the AAAP family shows that aphid amino acid transporter ACYPI008971 is orthologous to mammalian proton-coupled transporters SLC36A1, SLC36A2, SLC36A3, and SLC36A4 and *Drosophila melanogaster* amino acid transporter CG1139 (Price et al, 2011). SLC36A2 is an H⁺-coupled transporter, which means that this transporter is sensitive to pH (Thwaites et al, 2011). Research on rat SLC36A2 demonstrated that SLC36A2 is a pH-dependent and sodium-independent transporter that transports proline at low pH but gradually decreased its transport of proline at

increasing pH (Kennedy et al, 2005). Another study on *D. melanogaster* amino acid transporter CG1139 produced a similar result, showing that CG1139 can transport proline, alanine and glycine at pH 5.5 (Goberdhan et al, 2005). On the basis of previous functional characterization of ACYPI008971 orthologs I hypothesize that ACYPI008971 is an H⁺-coupled transporter that transports proline, alanine and glycine at pH 5.5.

Materials and Methods

Amino acid transporter RNA synthesis. The full-length *A. pisum* bacteriocyte amino acid transporter, ACYPI 008971 had previously been cloned into *E. coli* vector pcDNA3.1 (Invitrogen) by Dan Price (Price et al. 2014). I linearized ACYPI008971-pcDNA3.1 vector using restriction enzyme BamHI (Thermo) and used the linearized vector as the template for generating ACYPI008971 cRNA using a T7 mMACHINE kit (Ambion), following the manufacturer's instructions. I then used a Poly (A) Tailing Kit (Ambion) to poly-adenylated the cRNA encoding ACYPI008971.

Expressing ACYPI008971 in *Xenopus laevis* oocytes. The *Xenopus laevis* frogs used in this thesis were purchased from Nasco (Fort Atkinson, WI) and oocytes were surgically harvested from *X. laevis* frogs by Ben Sherman under the protocol approved by the University of Miami Animal Research Committee (Animal Welfare Assurance #A-3224-01, Protocol #13-056) in compliance with US National Institutes of Health guidelines. Healthy oocytes at stage V-VI, as judged by their round shape, clear color at each pole and absence of surrounding sheath cell, were chosen for microinjection of cRNA. Experimental oocytes were injected with 46 ng (1 ng/nL) of ACYPI008971

cRNA. While sham (negative control) oocytes were injected with 46 nL of DEPC water. For expressing the injected RNA, oocytes after microinjection were incubated in Barthe's buffer (in mM: 88 NaCl, 1 KCl, 2.4 NaHCO₃, 0.3 CaNO₃, 0.41 CaCl₂, 0.82 MgSO₄, 15 HEPES at pH 7.6, 100 µg/L amikacin, 50 µg/L ciprofloxacin, and 50 µg/L tetracycline) for 3 days before use.

Two-electrode voltage clamp electrophysiology (TEVC). The currents produced by the transporter ACYPI008971 were recorded by two-electrode voltage clamp (TEVC) using an automated parallel electrophysiology system (OpusExpress 6000A, Molecular Devices). Oocytes were placed in the running buffer, ND96 (in mM: 96 NaCl, 2 KCl, 1 CaCl₂, 1 MgCl₂, 5 HEPES, pH 7.4). Proline, alanine, glycine and glutamine at 2 mM and proline, alanine, glycine and leucine at six different concentrations (0.3mM, 1mM, 3mM, 10mM, 30mM and 100mM) were prepared fresh using pH 5.5 and pH 7.4 ND96 buffer on the day of recording. Glutamine and leucine were both used as negative controls. ACYPI008971 has small response to glutamine (Price et al, 2014) and almost no response to leucine (David Thwaites and Noel Edwards, unpublished uptake assay data). All amino acids were applied for 60 sec at a flow rate of 1.0 mL/min and washing in ND96 at the speed of 4.6 mL/min between each application. In order to allow the currents to return to baseline after applications, a longer washout time was introduced. The washout time between each application was increased according to the increased concentration of applied substrates. In the final protocol I used a 7 min wash between 0.3mM and 1mM, and the 1mM and 3mM applications, an 8 min wash between the 3mM and 10mM, a 9 min wash between 10mM and 30mM and finally, a 10 min wash

between the 30mM and 100mM applications. The electrodes were filled with 3M KCl and had resistances of 0.2–2.0 M Ω . The holding potential was -70 mV.

Data analysis. Quantification results were generated using pClamp software. Bar graphs were generated by normalizing data. Data normalization in this thesis contains two steps: one is calculating the mean of control group and the other is comparing each data in experimental group to the mean of control group I calculated at first step. Bar graphs were created by Prism 5.0c software. Error bar represents \pm SEM. Michaelis-Mentens kinetic (Figure 2.4) were generated by nonlinear regression using Prism 5.0c software. The values shown in Figure 2.4 are $V_{\max} \pm$ SEM and $K_m \pm$ SEM. All calculations were automatically executed by Prism 5.0c Software.

Results

The electrophysiology result showed that ACYPI008971 responds to 2mM proline, alanine and glycine (Figure 2.1) but has very little response to glutamine. However, I only obtained this result with one replicate because only two of 25 oocytes survived injection and only one of the two responded to the amino acid application. In order to trouble-shoot the high death rate of the oocytes and to improve data collection, I performed a series of experiments that involved manipulations that included decreasing the number of days for cRNA expression prior to TEVC, minimizing the time that oocytes were exposed to pH5.5 and increasing the working pH to pH7.4. These modifications did somewhat improve oocyte survival but had no significant impact on

the number of ACYPI008971 injected oocytes responding to the application of amino acid substrates (Table 2.1).

Following this first round of experiments it was necessary for me to synthesize more cRNA and thus, the dose dependent experiments were performed using a second batch of cRNA. I performed the dose dependent experiments using four amino acids: proline, alanine, glycine, and leucine as a negative control. Each amino acid was applied at six different concentrations: 0.3mM, 1mM, 3mM, 10mM, 30mM and 100mM. Consistent with the results I obtained from a single oocyte in the first experiment (Figure 2.1) ACYPI008971 responded to the application of proline, alanine or glycine and further, these responses intensified with increasing concentrations of all three amino acids (Figure 2. 2 A, B and C). On the other hand, ACYPI008971 showed an insignificant response to any concentration of leucine except at the highest concentration (100mM) (Figure 2. 2 D). However, sham oocytes responded in a similar way to the application of 100mM leucine. Quantification analysis of the TEVC traces demonstrated that ACYPI008971 responds to proline, alanine and glycine and that the magnitude of the response increases with increasing substrate concentration (Figure 2.3). Michaelis-Mentens kinetic analysis of ACYPI008971 responses indicated that this transporter has greater affinity to proline and alanine than that to glycine (Proline: K_m 3.762, Alanine: K_m 5.941, Glycine: K_m 46.37) (Figure 2.4 A, B and C). ACYPI008971 only responded to high concentrations of glycine (Figure 2.3 C and Figure 2.4 C). ACYPI008971 did not respond to leucine (Figure 2.4 D).

Discussion

Aphids cannot synthesize essential amino acids by themselves. Dietary supplementation of essential amino acid is critical for aphid growth and reproduction. *Buchnera*, the primary microbial symbiont of aphids (Hinde, 1971), can synthesize essential amino acids when the host aphid supplies the precursor amino acids (Thomas, et al, 2009; Price et al, 2014). Price et al (2014) demonstrated that glutamine was transported by *A. pisum* glutamine transporter 1 (ApGLNT1) from aphid hemolymph to bacteriocytes. In the cytoplasm of bacteriocytes, glutamine is converted to glutamate (Hansen and Moran, 2011) and glutamate can be the precursor for proline and alanine biosynthesis (Hansen and Moran, 2011; Shigenobu and Wilson, 2011). ACYPI008971 is an amino acid transporter located on the symbiosomal membrane (unpublished data generated by Honglin Feng). A member of the AAP family of amino acid transporters, it is likely that ACPYI008971 transports specific substrate(s) by way of symport with one specific ion (Price et al, 2011; Saier, 2000). Symport allows electrodes to detect the current change generated by ion influx across the membrane when a transporter responds to substrates, thereby facilitating identification of transporter substrates.

In the first experiment of this thesis only one replicate of ACYPI008971 responded to stimuli by 2 mM substrates (proline, alanine, glycine and glutamine) at pH5.5 in ND-96 buffer while the other ACYPI008971 injected oocytes were dead before or at the time of the experiment. The high rate of oocyte death could result from low pH (pH 5.5), long expression time (3 days) or dirty RNA. However, I reason that oocytes should survive at pH5.5 since previous experiments with orthologs of ACYPI008971 utilized a pH 5.5 buffer (Goberdhan et al, 2005; Kennedy et al, 2005)

and further the modification experiment I performed with ACYPI008971 at higher pH did not significantly increase the number of oocytes surviving (Table 1). While decreasing the number of expression days for ACYPI008971 RNA injected oocytes did improve the situation of low oocytes survival, it also reduced the magnitude of ACYPI008971 response to substrates (Table 1). Therefore, low pH and long expression days did not appear to be the actual cause of high rates of oocytes loss in the first set of experiments (Table 1).

Following the first round of experiments, I ran out of RNA and needed to synthesize a new batch of ACYPI008971 cRNA. The survival rate of the oocytes increased with the new batch of cRNA. My dose dependent experiment demonstrated that ACYPI008971 responds to proline, alanine and glycine at pH7.4 in ND96 buffer and further that the magnitudes of these responses increased when the higher substrate concentrations were applied (Figure 2.2). I quantified the responses by measuring the amplitude of each response and plotted the data as a bar graph. The bar graph showed that ACYPI008971 responded to different concentrations of proline, alanine and glycine and that the magnitude of the response increased with application of higher concentration substrates (Figure 2.3 A, B and C). The results of the first and second rounds of experiments suggest that ACYPI008971 can transport proline, alanine and glycine. On the other hand, Michaelis-Mentens kinetic analysis suggests that the affinity of ACYPI008971 differs across the three amino acids. Affinity represents the relationship between transporter and its specific substrates. Higher affinity of a substrate means it has higher change to occupy its specific transporter. The constant K_m value represents the substrate concentration when reaches to half of maximum velocity. K_m

indicates the affinity between transporter and its specific substrate. The lower K_m , the higher affinity. In this case, ACYPI008971 has similar affinity for proline ($K_m 3.762 \pm 0.9931$) and alanine ($K_m 5.941 \pm 2.2460$) but lower affinity for glycine ($K_m 46.37 \pm 11.27$) compared to proline and alanine.

Chapter 2 Tables

	Expression time	Total RNA (Volume)	Working pH
Experiment 1	3 days	46 ng (46 nL)	pH5.5
<p>There are only two oocytes survive in total 25 injection (n=25). The survival rate of the oocyte is 8%. Only one oocyte response to alanine, glycine, proline and glutamine.</p>			
Experiment 2	2 days	46 ng (46 nL)	pH5.5
<p>26 oocytes survive (n=40) after two days expression. The survival rate of the oocyte is 65%. However, no oocytes showed response on proline, alanine and glycine.</p>			
Experiment 3	3 days	60 ng (50 nL)	pH5.5+pH7.4 washout
<p>18 oocytes survive (n=40) after three days expression. The survival rate of the oocyte is 45%. pH 5.5 buffer applied before each application of substrates then washout with pH7.4 buffer. 18 oocytes survive at the recording showing the response when applying proline (n=4) and alanine (n=3) and only at high concentration of glycine (n=4). No significant response on glutamine (n =4) and β-alanine (n=3).</p>			
Experiment 4	3 days	60 ng (50 nL)	pH5.5+pH7.4 washout
<p>8 oocytes survive (n=35) after three days expression. The survival rate of the oocyte is 23%. Similar experiment strategy, pH 5.5 buffer presented before and after each application of substrates. After that, oocytes would washout with pH7.4 buffer. Only proline (n=8) tested in this protocol.</p>			

Table 2.1 Troubleshooting experiments. Experiment 1 is the first experiment that yielded a lot of oocyte death prior to electrophysiology recordings. The experiments are numbered in the order of I performed them.

Chapter 2 Figures

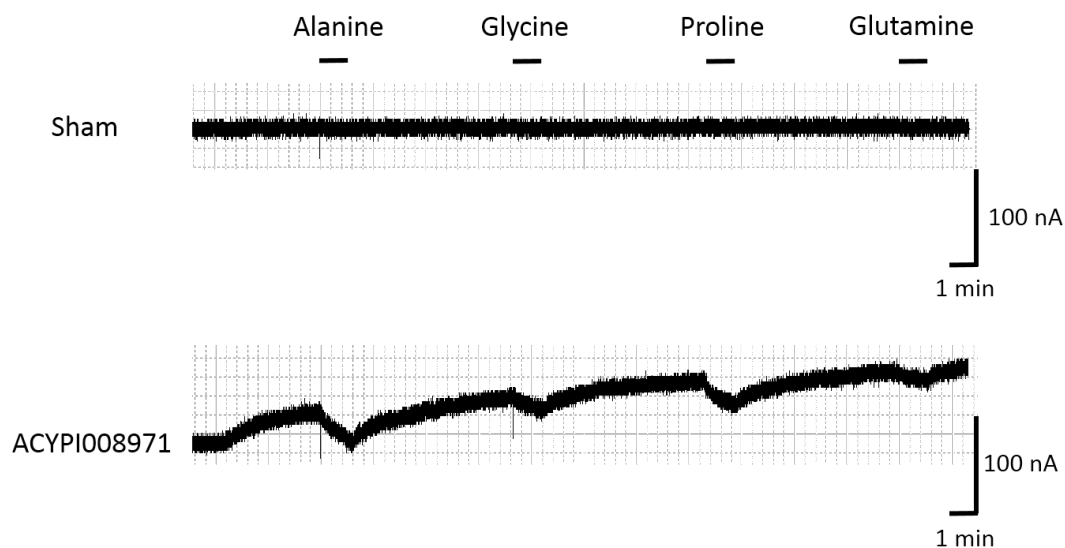


Figure 2.1 ACYPI008971 (8971) response to 2mM alanine, glycine proline and glutamine at pH 5.5 in ND-96 buffer. The experimental oocyte was injected with 46 ng (1 ng/nL) of ACYPI008971 cRNA and incubated for 3 days at 17°C, while the sham oocytes were injected with 46 nL of DEPC water. Glutamine was included in this experiment as a negative control (see Price et al, 2014). The response of ACYPI008971 to proline and alanine was stronger than its response to glycine. The sham oocytes did not respond to any of the four. Only one oocyte gave this result, all other oocytes injected with ACYPI008971 died before or at the time of the electrophysiology recording. Subsequent troubleshooting suggested that the high death rate of oocytes in this experiment resulted from dirty ACYPI008971 cRNA.

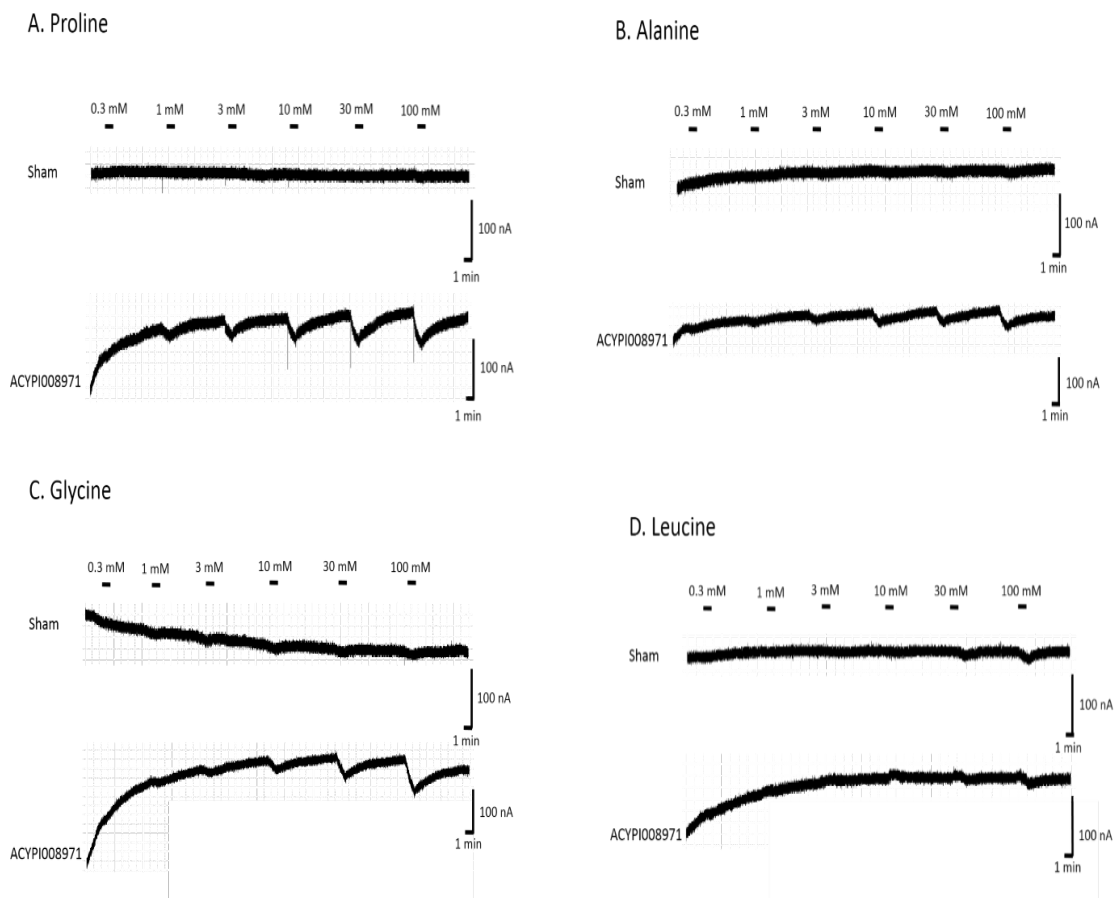


Figure 2.2 Dose-dependent experiment with (A) proline, (B) alanine, (C) glycine and (D) leucine. Six different concentrations of amino acid were applied: 0.3mM, 1mM, 3mM, 10mM, 30mM and 100mM. ACYPI008971 showed a concentration dependent response to proline, alanine and glycine. The amount of cRNA injected was 46ng (1ng/nL), while sham oocytes were injected with 46 nL of DEPC water. Injected oocytes were incubated for 3 days before recording. Replicates for each group: (A) proline n=13, Sham-pro n=17 (B) alanine n=11, Sham-ala n=13 (C) glycine n=10, Sham-gly n=7 (D) leucine n=11, Sham-leu n=10.

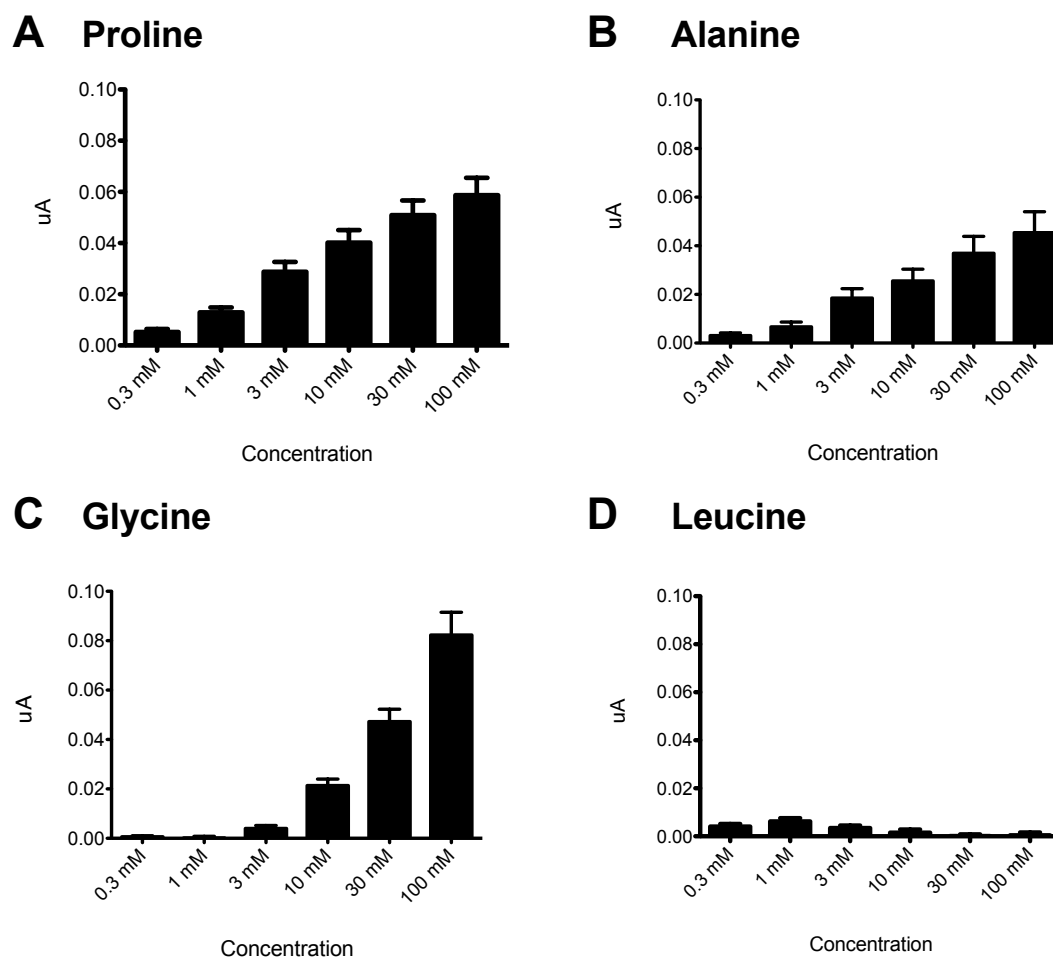


Figure 2.3 Quantification of ACYPI008971 response to proline, alanine, glycine and negative control leucine. All the bars are normalized to the control (Sham). (A) Proline had 13 ACYPI008971-injected replicates and 17 sham replicates. (B) Alanine had 11 ACYPI008971-injected replicates and 13 sham replicates. (C) Glycine had 10 ACYPI008971-injected replicates and 7 sham replicates. (D) Leucine had 11 ACYPI008971-injected replicates and 10 sham replicates. Each value is mean \pm SEM.

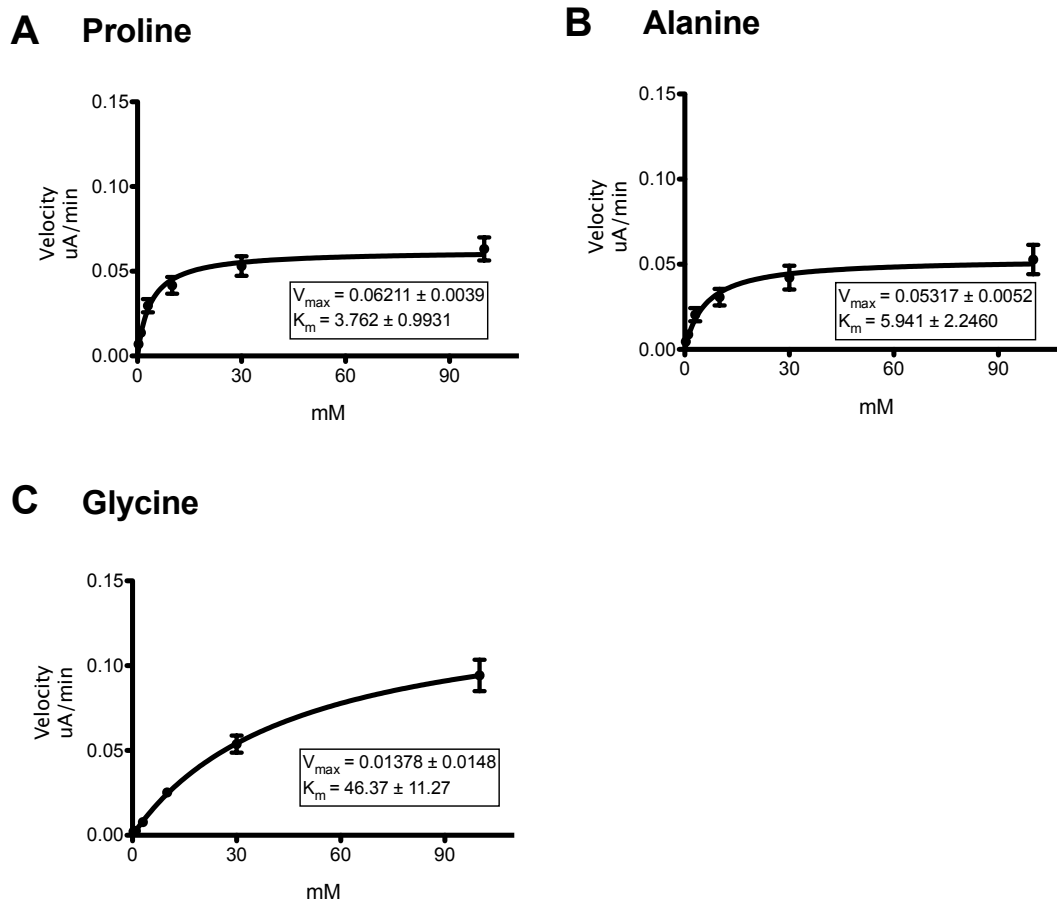


Figure 2.4 Michaelis-Mentens kinetics of the response of ACYPI008971 to three different amino acid substrates. All three amino acids responses were fit to the equation of Michaelis-Mentens. K_m and V_{max} for all three curves were calculated using Prism 5.0. (A) Proline: 13 ACYPI008971-injected replicates and 17 sham replicates; $r^2 = 0.61$, 95% confident intervals for $V_{max} = 0.054$ - 0.070 and $K_m = 1.781$ - 5.742 . (B) Alanine: 11 ACYPI008971-injected replicates and 13 sham replicates; $r^2 = 0.51$, 95% confident intervals for $V_{max} = 0.043$ - 0.064 and $K_m = 1.452$ - 10.43 . (C) Glycine: 10 ACYPI008971-injected replicates and 7 sham replicates; $r^2 = 0.86$, 95% confident intervals for $V_{max} = 0.108$ - 0.168 and $K_m = 23.80$ - 68.94 . These plots were generated by nonlinear regression using Prism 5.0c software.

Chapter 3: Conclusion

Although phloem sap is a favorable food source for aphids since the phloem sap of most of plants contains high carbon, nitrogen in the form of free amino acids and is free of toxins and other feeding deterrents, phloem sap presents aphids with a “nitrogen barrier” that they must overcome (Douglas, 2006). Bacterial endosymbionts have been living in aphids for at least 160 million years (Moran et al, 1993). Aphids can synthesize non-essential amino acids and these non-essential amino acids work as the precursors of essential amino acids biosynthesis inside the endosymbiont *Buchnera* (International Aphid Genomic Consortium, 2010; Shigenobu and Wilson, 2011). As the result of host/endosymbiont co-evolution, symbionts have reduced genome sizes, which are associated with loss of genes that include the genes for non-essential amino acid biosynthesis; as such these endosymbionts have lost the ability of surviving outside the host (Gil et al, 2002; Pérez-Brocal et al, 2006; Smith, 1991; Akman et al, 2002; Shigenobu et al, 2000; Moran, 2002). Recently it has been revealed that host aphids can regulate the output of essential amino acids from *Buchnera* by controlling the input of non-essential amino acid precursors (Price et al, 2014).

Price et al (2014) generated an amino acid metabolic pathway by functional characterizing the glutamine transporter ApGLNT1 (located on bacteriocyte cell membrane) but the identity of downstream transporters, which are located on the symbiosomal or *Buchnera* inner and outer cell membrane is still not known. This thesis, via two-electrode voltage clamp (TEVC) electrophysiology, identified the function of one amino acid transporter ACYPI008971, which is located on symbiosomal or *Buchnera* inner and outer cell membrane. TEVC electrophysiology measures the current

change caused by ion influx in response to substrates. Here I showed that, ACYPI008971 responds to application of proline, alanine and glycine.

Based on the response of ACYPI008971 to proline, alanine and glycine, I propose an advanced model of amino acid metabolism in *A. pisum*. As proposed by Price et al (2014), glutamine is transported from hemolymph by ApGLNT1 and transformed to glutamate in the cytoplasm of bacteriocytes. That glutamate then serves as the precursor for proline and alanine biosynthesis. Proline and alanine are then transported by ACYPI008971 from the cytoplasm of bacteriocytes to *Buchnera* (Figure 3.1). On the other hand, both *A. pisum* and *Buchnera* are capable of glycine biosynthesis (Shigenobu and Wilson, 2011). In *A. pisum* alanine serves as the precursor for glycine biosynthesis (Hansen and Moran, 2011), while in *Buchnera* serine serves as the metabolic precursor for glycine biosynthesis. Both aphid (in the cytoplasm of bacteriocytes) and *Buchnera* synthesize glycine causing the two possible transportation directions by ACYPI008971 while proline and alanine have only one direction that is from bacteriocyte cytoplasm to *Buchnera* (Figure 3.1).

Macdonald et al (2012) generated an overview of metabolic pathway in aphid/*Buchnera* symbiosis by flux balance analysis (FBA) that can calculate the optimal biomass production. Their metabolic model (iSM199), improved a previous model iSM197 (Macdonald et al, 2011), recalculated the amino acid stoichiometries of the *Buchnera* biomass reaction by using the data from *Buchnera* quantitative proteome (Macdonald et al, 2012, Poliakov et al. 2011). The iSM 199 model generated the flux unit of each metabolite across to symbiotic interface from aphid to *Buchnera*. The input (from aphid to *Buchnera*) in flux units of proline and alanine was 2.08 and 4.77,

respectively while the output (from *Buchnera* to aphid) of glycine was 0.84 (Macdonald et al, 2012). Alanine has higher flux than proline and glycine has lowest flux among three, suggesting that the aphid supplies more alanine than proline to *Buchnera* while *Buchnera* release less glycine (Macdonald et al, 2012). I have generated the Michaelis-Mentens kinetic on the response of ACYPI008971 to three different amino acid substrates and the result showed that ACYPI008971 has similar affinity and maximum velocity on proline and alanine but lowest on glycine (Figure 2.4). The similar affinity of ACYPI008971 on proline and alanine cannot explain why aphid supplying more alanine than proline. Perhaps, the similar affinity on proline and alanine is caused by the limitation of TEVC electrophysiology since it only can test the ion flux but not the actual transportation ability of ACYPI008971. Future work using isotope uptake assays will be critical for determining the relative ability of ACYPI008971 to transport proline versus alanine.

Functional characterization of ApGLNT1 provided a mechanism by which the host can regulate amino acid biosynthesis in *A. pisum/Buchnera* symbiosis (Moran et al, 2003; Thomas et al, 2009; Price et al, 2014). In this thesis I advanced understanding of the coordination of host/symbiont metabolism through functional characterization of a downstream amino acid transporter ACYPI008971, a transporter that localizes to the symbiosomal membrane or the *Buchnera* inner and outer membranes. This advanced understanding of amino acid metabolism can help to elucidate the integration of amino acid metabolism between host and symbiont.

In this thesis, four amino acids, proline, alanine, glycine and leucine, were applied to ACYPI008971 separately. The possibility of the competition among these

amino acids has not been tested and generates questions about substrate competition in bacteriocytes. On the other hand, there are few studies on the function of amino acid transporter ACYPI008971 orthologs in sap-feeding insect. Characterizing the function of ACYPI008971 orthologs in other sap-feeding insects presents a feasible direction for future work on this transporter.

Chapter 3 Figure

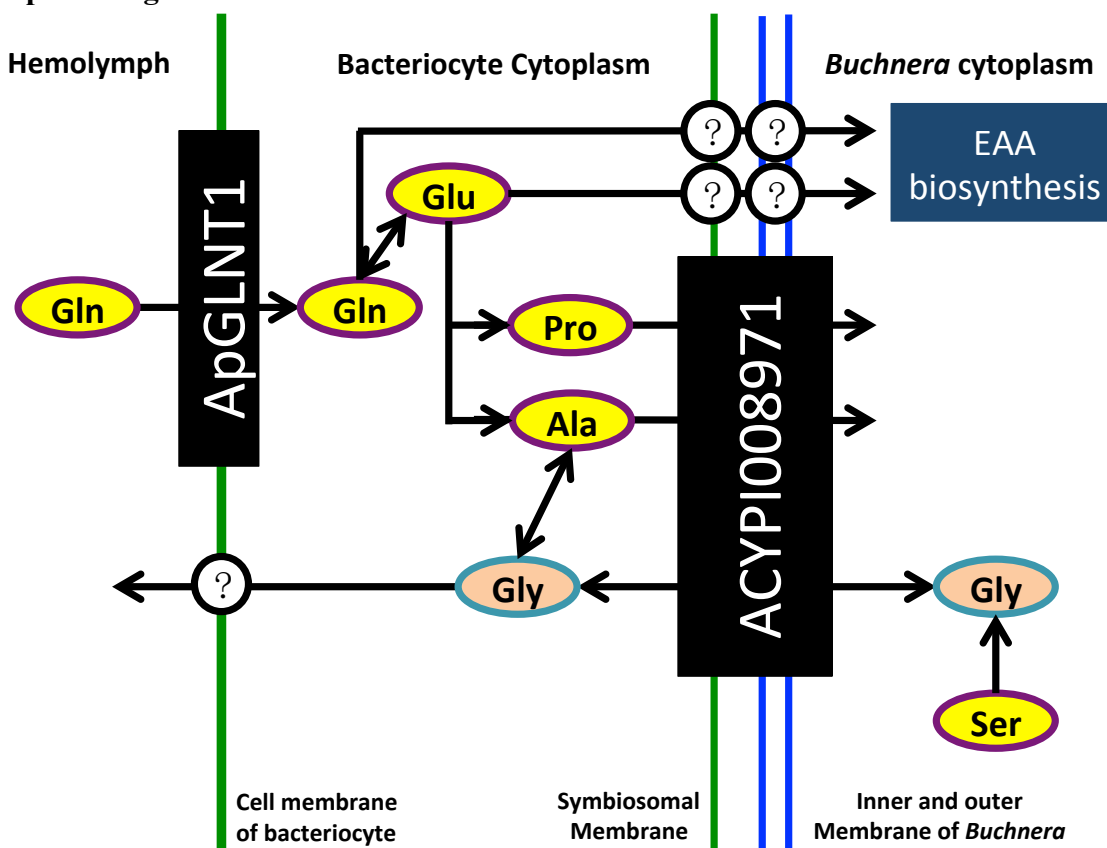


Figure 3.1 Model of amino acid metabolism pathway in aphid/*Buchnera* symbiosis. Glutamine (Gln) is transported by ApGLNT1 where it feeds into the GOGAT cycle (figure not shown) to produce glutamate (Glu), the metabolic precursor of proline (Pro) and alanine (Ala) biosynthesis. These two amino acids, Pro and Ala, are then transported by ACYPI008971 from bacteriocyte cytoplasm to *Buchnera*. Glycine (Gly), on the other hand, can be transported in both directions, from host to endosymbiont and from endosymbiont to host. Two aphid derived membranes are shown as green lines; they are the cell membrane of bacteriocytes (thick green line) and the symbiosomal membrane (thin green line). *Buchnera* inner and outer membranes are shown in blue.

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