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Symbiotic Microorganisms in Aphids (Homoptera, Insecta): A Secret of One Thriving Insect Group

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Most, if not all, aphids harbor intracellular bacterial symbionts, called *Buchnera*, in their bacteriocytes, huge cells differentiated for this purpose. The association between *Buchnera* and aphids is so intimate, mutualistic and obligate that neither of them can any longer reproduce independently. *Buchnera* are vertically transmitted through generations of the host insects. Evidence suggests that *Buchnera* were acquired by a common ancestor of aphids 160-280 million years ago, and have been diversified, since then, in parallel with their aphid hosts. Molecular phylogenetic analyses indicate that *Buchnera* belong to the g subdivision of the Proteobacteria. Although *Buchnera* are close relatives of *Escherichia coli*, they contain more than 100 genomic copies per cell, and their genome size is only one seventh that of *E. coli*. The complete genome sequence of *Buchnera* revealed that their gene repertoire is quite different from those of parasitic bacteria such as *Mycoplasma*, *Rickettsia* and *Chlamydia*, though their genome sizes have been reduced to a similar extent. Whereas these parasitic bacteria have lost most genes for the biosynthesis of amino acids, *Buchnera* retain many of them. In particular, *Buchnera*'s gene repertoire is characteristic in the richness of the genes for the biosynthesis of essential amino acids that the eukaryotic hosts are not able to synthesize, reflecting a nutritional role played by these symbionts. *Buchnera*, when housed in the bacteriocyte, selectively synthesize a large amount of symbionin, which is a homolog of GroEL, the major stress protein of *E. coli*. Symbionin not only functions as molecular chaperone, like GroEL, but also has evolutionarily acquired the phosphotransferase activity through amino acid substitutions. Aphids usually profit from *Buchnera*'s fuction as a nutritional supplier and, when faced with an emergency, consume the biomass of *Buchnera* cells as nutrient reserves.

All organisms do not live by themselves. The life of one species is necessarily related to that of others in a variety of ways. For example, one of the principal ecological niches of microorganisms is the inside of eukaryotic cells. These interspecific relationships will greatly influence not only physiology but also evolution of the organisms involved in them. Among others, the relationships between homopteran insects such as aphids, whiteflies, psyllids, scales, mealybugs, leafhoppers and planthoppers and their microbial endosymbionts are particularly noteworthy in that most of them are obligate mutualism (Buchner, 1965; Ishikawa, 1989; 1996). All these insects feed on nutrient-deficient plant sap, and appear to fortify their diet with the metabolic products of mutualistic microorganisms (Houk and Griffiths, 1980).

Among homopterans, aphids (family Aphididae) are remarkable in particular for their terrible fecundity and unique life style, as well as species diversity with broad ecological niches. Indeed, our earlier studies demonstrated that aphids form an exceptional insect group in terms of molecular biology (Ishikawa, 1976; Ogino et al., 1990; Kwon et al., 1991; Amako et al., 1996). It is beyond question that aphids fecundity, which is exceptionally high as metazoa, is due to their unique mode of reproduction, thelytoky or female-producing parthenogenesis. However, it is not widely known that it is their symbiotic bacteria that actually facilitate this reproductive system to operate efficiently. In this article, I describe a brief overview of endo-symbionts of aphids to begin with, and next focus upon *Buchnera*, intracellular symbiotic bacteria that play a pivotal role in the physiology, and probably, evolution of these insects.

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Endosymbionts of Aphids

Buchnera

About 4,400 species of aphids have been identified,

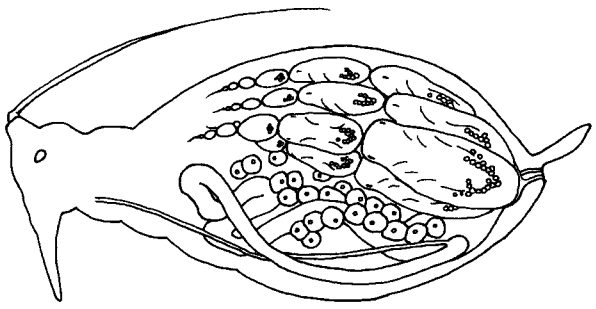

Fig. 1. Bacteriocytes in the aphid body (figure drawn by T. Sasaki). Tens of bacteriocytes are located around the gut. Developing embryos contain small-sized bacteriocytes.

most of which inhabit the temperate zone. Most, if not all (see later), of them harbor intracellular bacteria, designated *Buchnera*, in the bacteriocyte, or mycetocyte, a huge cell that is located in the fat body (Fig. 1). In the case of the pea aphid, *Acyrthosiphon pisum*, an adult contains several tens of bacteriocytes whose cytoplasm is packed with many *Buchnera* cells. The size of the bacteriocyte is so large, more than 100 μm in diameter, and the density of the *Buchnera* cell within it is so high, that the average density of this bacterium in the whole aphid body is about 10^9/ml, a density comparable to that of *Escherichia coli* cells in the medium after an overnight culture. The academic name *Buchnera aphidicola* was coined by Baumann's group in Davis to call intracellular symbionts of the aphid bacteriocyte collectively (Munson et al., 1991) after the name of Paul Buchner who studied extensively symbiosis and parasitism between animals and microorganisms (Buchner, 1965). Nowadays, however, only the genus name of *Buchnera* is widely accepted because it is evident that *Buchnera* the symbionts are as diversified as their host aphids.

Sequence comparisons of many genes of *Buchnera* have indicated that these bacteria belong to the γ subdivision of the Proteobacteria, and are phylogenetically close to *E. coli* in particular (Baumann et al., 1995).

Secondary symbionts

Sometimes, aphids harbor some more intracellular bacteria in addition to *Buchnera*. These bacteria are called secondary symbionts, which are housed in cells other than the bacteriocyte harboring *Buchnera*. Whereas *Buchnera* are nearly spherical in shape, the shape of secondary symbionts is various and can be rodlike or fibrous. Unlike *Buchnera* that are monophyletic (see later), secondary symbionts are various in origin and belong to many distinct groups of bacteria, suggesting that their symbioses with aphids began independently from each other after the speciation of the host insect species. Even within a single species of aphids, the presence or absence of secondary symbionts is sometimes different due to strain and biotype. The number of secondary symbionts in each aphid is much less than that of *Buchnera*. All these observations suggest that the symbioses of secondary symbionts with aphids are relatively unstable, and that their initiation is a more recent event than that of the symbiosis between *Buchnera* and aphids. Indeed, compared with those of *Buchnera*, the cell walls of secondary symbionts are considerably thicker, and reminiscent of those of free-living bacteria. Gene expression pattern of secondary symbionts is also similar to that of free-living bacteria rather than that of *Buchnera* (Fukatsu and Ishikawa, 1993), which selectively express a single gene (see in section 6; Ishikawa, 1982).

Yeast-like symbionts

There are a few exceptional species in aphids, which do not have any intracellular bacterial symbiont. They do not have bacteriocytes either, and instead, contain yeast-like cells in the intercellular space of the fat body (Buchner, 1965). Electron micrography demonstrated the presence of cell organelles, such as nucleus and mitochodria in this cell, indicating that this is a eukaryotic extracellular symbiont. The exceptional aphid species containing these yeast-like symbionts (YLS) belong to four genera in the tribe Cerataphidini (Fukatsu et al., 1994).

About 30 species in 10 genera of Cerataphidini are divided into the two groups, one with YLS and the others with *Buchnera*. No single species harbors both YLS and *Buchnera*. All the aphid species belonging to the tribes other than Cerataphidini do not contain YLS but *Buchnera*. Whereas YLS morphologically resemble budding yeasts, a molecular phylogenetic study based on 18S rDNA suggests that they are members of the Ascomycota (Fukatsu and Ishikawa, 1996). It is particularly noteworthy that YLS are phylogenetically close to *Cordyceps*, or vegetative wasps. It is a possible scenario that one of the parasitic fungi gave up its pathogenicity to be a mutualistic symbiont. In view of the distribution of *Buchnera* and YLS, it is highly likely that a common ancestor of extant aphids once acquired *Buchnera*, and that later on only in a small group of its descendants, *Buchnera*, the universal symbionts were taken over by YLS. What caused such substitution of symbionts is a sheer enigma. The fact that all the Cerataphidini aphids, including those with YLS, are eusocial insects having the soldier caste, forming characteristic galls on their host plants (Aoki, 1977) may provide a clue to solve this enigma. As described later, the presence of *Buchnera* is nutritionally essential to aphids. Are YLS still more beneficial to the exceptional group of aphids than is the common benefactor, *Buchnera*, which all the other aphids share?

Origin and Evolution of *Buchnera*

Among the three families including Aphididae, Adelgidae and Phylloxeridae that comprise the superfamily

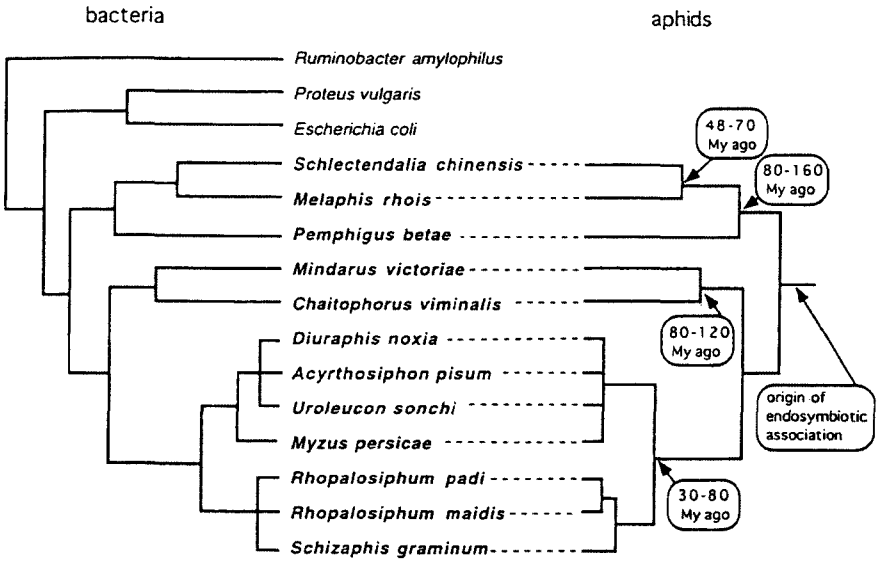

Fig. 2. Phylogeny of *Buchnera* and that of the corresponding aphid hosts. The bacterial phylogeny is based on 16S rDNA sequences; the aphid phylogeny is based on morphological traits. Taxa within *Buchnera* are represented by names of their aphid hosts. Dates are estimated from aphid fossils and/or biogeography (Moran and Baumann, 1994).

Aphidoidea, members of the Phylloxeridae do not contain *Buchnera*. This is probably because they ingest plant cytosol instead of phloem sap that Aphididae and Adelgidae ingest. Compared with phloem sap, plant cytosol is a nutritionally better balanced diet, which may enable the Phylloxeridae to do without *Buchnera*, a nutritional supplement (Douglas, 1998).

Gut microbe similar to Buchnera

Culture of honeydew, the excreta of aphids, spread over nutritional agar demonstrated that pea aphids, *A. pisum*, contain several species of gut microbes (Harada and Ishikawa, 1993). Whereas most of them were identified to be bacterial exoparasites of plants, one was an unidentified bacterium, which was designated *Erwinia aphidicola* (Harada et al., 1997). *Erwinia aphidicola* belongs to the γ subdivision of the Proteobacteria, thus it is phylogenetically close to both *E. coli* and *Buchnera*. Sequence identity of 16S rDNA between *Erwinia aphidicola* and *Escherichia coli*, between *E. aphidicola* and *Buchnera*, and Between *E. coli* and *Buchnera* was 96%, 87%, and 89%, respectively. Thus, it is possible that *E. aphidicola*, a gut microbe, and *Buchnera* share a direct ancestor in common.

Molecular phylogeny

Moran et al. (1993) constructed a molecular phylogenetic tree of *Buchnera* species from various aphids based on 16S rDNA sequences. Also, they constructed a phylogenetic tree of their host aphids based on their morphological traits. Upon comparison of the two trees, it was found that the phylogeny of *Buchnera* is completely concordant with that of their host aphids (Fig. 2). This concordance implies a single original infection in a common ancestor of aphids, followed by cospeciation of aphids and *Buchnera*. The universal distribution of *Buchnera* species among their host aphids thus reflects parallel cladogenesis through consistent, long-term vertical transmission from the mother to the daughter of aphids.

Ancient aphids that were preserved in amber give us a clue on dates when aphid groups diverged from each other. The dates correspond to paticular nodes in the phylogenetic tree of aphids, and thus those of the phylogenetic tree of *Buchnera*. Based on these data, the base substitution rate of 16S rDNA was calculated to be 0.02-0.04 per nucleotide site per 100 million years. This substitution rate, together with other circumstantial evidence, suggests that acquisition of *Buchnera* by a common ancestor of aphids dates back to 160-280 million years ago (Moran and Baumann, 1994).

Buchnera and Nutritional Physiology of Aphids

Aphids, when injected with an antibiotic refampicin, give birth to next generation without *Buchnera*. Such aposymbiotic aphids show retarded growth and usually die without producing progeny, suggesting that aphids are profoundly dependent on these intracellular symbionts. That *Buchnera* also benefit from this association is usually assumed because their habitat is restricted to the bacteriocyte, an insect cell (Ishikawa, 1989; Douglas, 1989). Whereas isolated *Buchnera* cells can be maintained outside bacteriocyte for a short period, no attempts to grow them in culture media have been successful. In the course of studies

on this obligatory and mutualistic symbiosis, how *Buchnera* benefit the host has consistently intrigued researchers. Chemically-defined diets have been used extensively to explore the nutritional requirements of aphids.

Cholesterol

Cholesterol was once suspected to be one of the nutrients that are provided by *Buchnera*. Insects, in general, are incapable of *de novo* synthesis of sterols, although cholesterol is an indispensable substrate for the synthesis of molting hormones. Because several species of aphids can be maintained for many generations on synthetic diets that lack sterols, they were looked upon as an exception (Dadd and Mittler, 1966; Srivastava and Auclair, 1971). Regarding this, it was suspected that *Buchnera* synthesized sterols and supplied them to the host. Whereas some results seemed to provide evidence for such a contribution (Houk et al., 1976; Griffiths and Beck, 1977), it was suggested that aphids are able to convert phytosterols into cholesterol (Campbell and Nes, 1983). If aphids in nature can exploit phytosterols, as other phytophagous insects, there should be no need for them to harbor sterol-producing symbionts. Moreover, sterol synthesis is not an attribute of prokaryotes, but that of eukaryotes. As expected, our genome analysis showed that the *Buchnera* genome does not contain genes for sterol synthesis (Shigenobu et al., 2000).

Vitamins

Phytophagous insects, in general, require vitamin B-complex as essential nutrients (Dadd et al., 1967). However, several aphids survive without some vitamin B-complex in synthetic diets. Using differential cDNA display and quantitative RT-PCR, Nakabachi and Ishikawa (1997) demonstrated that one of the *Buchnera* genes that are actively expressed codes for the riboflavin synthase b chain. Since all animals, including insects, are unable to synthesize the isoalloxazine ring (Miller and Silhacek, 1995), this result prompted us to examine the effect of dietary riboflavin on the performance of symbiotic and aposymbiotic aphids. As a result, dietary riboflavin exhibited contrasting effects on the symbiotic and aposymbiotic aphids. Whereas the dietary vitamin was slightly detrimental to symbiotic aphids, it was essential to aposymbiotic aphids and improved their performance remarkably (Nakabachi and Ishikawa, 1999). These results provide conclusive evidence that *Buchnera* synthesize riboflavin and supply to the host insect.

Amino acids

Aphids, like other animals, are believed to require 10 essential amino acids; arginine, histidine, isoleucine, leucine, lysine, methionine, threonine, tryptophan, valine and phenylalanine. Phloem sap, the sole diet of aphids, is rich in carbohydrates, but notoriously poor in nitrogenous compounds (Douglas, 1993). Accordingly, it had long been an enigma that aphids are so prosperous with extraordinarily high fecundity, notwith standing feeding on such a poor diet. Many lines of work provide evidence that their symbiotic system plays a crucial role in tiding over this nutritional difficulty of aphids (Douglas, 1998). However, until recently a precise mechanism as to how the symbiotic system makes up for the dietary deficiency in amino acids has not been understood. By analyzing nitrogenous compounds in honeydew, the excreta, of *A. pisum*, Sasaki et al. (1990) showed that aphids, whether symbiotic or aposymbiotic, excrete no detectable amount of uric acid, a common nitrogenous waste product of many insect species, and that the major excretory nitrogenous compounds are amino acids. They showed also that aposymbiotic aphids excrete more amounts of asparagine and glutamine than symbiotic ones, suggesting that these amino acids, which are two most abundant nitrogenous compounds in phloem sap, are utilized more efficiently by symbiotic aphids than by aposymbiotic aphids. These findings primed extensive studies on the metabolism of glutamine and asparagine in aphids using chemically-defined diets.

Nitrogen recycling

When aposymbiotic aphids were reared on a synthetic diet mimicking phloem sap in amino composition, glutamine and asparagine were the most abundant amino acid constituents in their honeydew. By contrast, symbiotic aphids on the same diet excreted these amides only in trace amounts, confirming the involvement of *Buchnera* in the utilization of these amides. While the omission of these amides from the synthetic diet severely retarded the growth and reproduction of symbiotic aphids, it caused no effects on the performance of aposymbiotic aphids, or somewhat improved it. It was especially noteworthy that aposymbiotic, but not symbiotic, aphids excrete a large amount of glutamine even when kept on diets not containing either of the two amides. These results suggest that aphids not only ingest the amides, but also synthesize them, and that symbiotic, but not aposymbiotic, aphids are capable of reutilizing of these amino acids (Sasaki and Ishikawa, 1993). This possibility was tested by keeping aphids on diets containing [ε-^{15}N] glutamine. It was shown that the ^{15}N level in their honeydew remained very low over several days in symbiotic aphids even though aphids were kept on the labeled diet throughout this period. By contrast, the ^{15}N level in the honeydew of aposymbiotic aphids on the same diet gradually increased. In addition, aposymbiotic aphids excreted large amounts of glutamine and asparagine that were highly labeled with ^{15}N (Sasaki et al., 1993). It was also demonstrated that symbiotic, but not

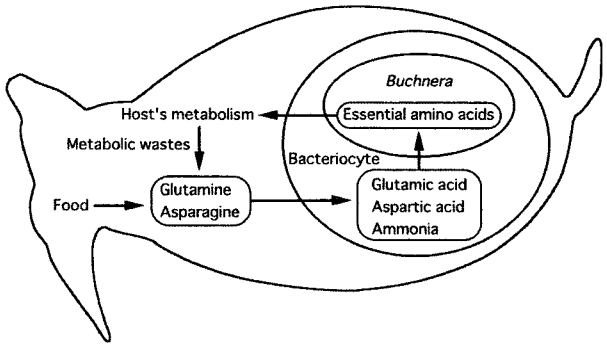

Fig. 3. Nitrogen recycling in the symbiotic system in aphids (figure drawn by T. Sasaki).

aposymbiotic, aphids were able to incorporate ^{15}N into many essen- tial amino acids (Sasaki et al., 1991). All these results, taken together, provide convincing evidence that symbiotic aphids are capable of recycling the amino group via glutamine, and possibly asparagine (Febvay et al., 1995).

Further studies on the mechanisms of nitrogen recycling were performed using bacteriocytes and *Buchnera* cells isolated from pea aphids, *A. pisum*. The results indicated that glutamine taken up by bactriocytes is hydrolyzed into glutamate and ammonia before being further taken up by *Buchnera*. Incubation of bacteriocytes with [ε-^{15}N] glutamine gave a rise to [^{15}N] glutamate in the medium, suggesting that at least a portion of ammonia split from glutamine is assimilated into glutamate probably through a reaction with 2-oxoglutarate. When isolated *Buchnera* cells were incubated with [^{15}N] glutamate, the following amino acids were found highly labeled: alanine, aspartate, glutamine, isoleucine, leucine, phenylalanine, proline and valine (Sasaki and Ishikawa, 1995). It is now evident that in aphids most, if not all (Douglas, 1998), essential amino acids are synthesized by their symbiotic system with *Buchnera*, using glutamine, both ingested and recycled, as a substrate (Fig. 3). Recently, it was suggested that some of the enzymes resposible for upgrading glutamate in *Buchnera* are induced only in the symbiotic system of young aphids, and that asparagine taken up by bacteriocytes is deaminated and converted into glutamate before being taken in by *Buchnera* (Nakabachi and Ishikawa, 1997).

As described in the later section, the genome analysis confirmed that *Buchnera* retain virtually all the genes necessary for the synthesis of the essential amino acids that the host cannot produce (Shigenobu et al., 2000). In considering that glutamine and asparagine are the two most abundant nitrogenous compounds in phloem sap on which aphids feed (Sasaki et al., 1990), it is quite reasonable that aphids have evolved a symbiotic system that makes the most of these amide amino acids.

Genome of *Buchnera*

It is widely accepted that ancestors of eukaryotic cell organelles, such as mitochondria and plastids, are free-living bacteria that got associated intracellularly with primitive eukaryotic cells about 1 billion years ago (Margulis, 1970; Gray et al., 1999). The date suggests that the aphid-*Buchnera* symbiosis is about 25% as old as associations of eukaryotes with these organelles. In the case of organelles, ancestral genomes have been greatly reduced and reorganized through gene loss and transfer from organelles to nucleus (Gray et al., 1999). As a result, organellar genomes at present differ greatly from those of related free-living bacteria. This tempted us to estimate the genome size of *Buchnera* from the pea aphid *A. pisum* using pulse-field gel electrophoresis (PFGE) (Charles and Ishikawa, 1999).

Genome size

To estimate the genome size of *Buchnera*, we took advantage of the extremely low G+C content of their genomic DNA (Ishikawa, 1987), which is probably due to a strong AT pressure (Sueoka, 1988) imposed on most intracellular bacteria (Moran, 1996). When digested with restriction endonucleases with recognition sequences rich in G and C, the *Buchnera* DNA gave rise to fragments whose sizes were suitable for analyses on PFGE. The restriction endonuclease recognizing the sequence conserved in rDNAs linearized the genomic DNA without fragmentation, suggesting that the genome is a closed circular molecule containing a single copy of rDNA, as suggested previously (Baumann et al., 1995). The genome size of *Buchnera*, estimated as the sum of the sizes of restriction fragments, was about 655 kb (Charles and Ishikawa, 1999). Sequence analysis of the *Buchnera* genome, to be described later, demonstrated that the exact size is 641 kb (Shigenobu et al., 2000). This represents the smallest of the completely sequenced genomes, except for that of *Mycoplasma genitalium* (580 kb) (Fraser et al., 1995). The slight overestimation of the size by PFGE was probably due to the high A+T content of the *Buchnera* DNA, which interferes with the mobility of large DNA fragments on PFGE (Pyle et al., 1988).

Comparison of sequences of many genes clearly indicates that *E. coli* is the phylogenetically closest bacterium to *Buchnera* (Clark et al., 1999). Consequently, the result that the genome size of *Buchnera* is only a seventh that of *E. coli* (4.67 Mb) suggests that *Buchnera* have lost many of the genes they shared with *E. coli* since divergence from a common progenitor. In other words, the genome shrinkage found in *Buchnera* is most likely a direct consequence of their intracellular symbiosis over 200 million years.

Genomic copy number

Despite a dramatic reduction in the genome size, *Buchnera* cells are much larger in volume than *E. coli* cells, and divide very slowly. These observations led us to suspect that, unlike *E. coli* and many other bacteria, *Buchnera* can contain many copies of the genome in a single cell. This possibility was tested using three different methods, namely dot-blot hybridization, fluorimetry using a video- intensified microscope photon-counting system, and real-time quantitative PCR (Komaki and Ishikawa, 1999; 2000). As a result, we obtained convincing evidence that each cell of *Buchnera* contains 120 genomic copies on average.

A dramatic reduction in the genome size (Charles and Ishikawa, 1999) accompanied by an exceptional increase in the genomic copy number (Komaki and Ishikawa, 1999) is reminiscent of eukaryotic cell organelles, such as mitochondria and plastids (Gray et al., 1999). Loss of the ability to divide outside the eukaryotic cell is also a common attribute of *Buchnera* and these organelles. It is possible that these changes are an inevitable consequence for the prokaryotes that have been housed in eukaryotic cytoplasm for an evolutionary length of time. The prolonged intracellular life may cause prokaryotes to abandon their common raison d'être of proliferating rapidly by frequent cell division. Notwithstanding this, if they still retain the ability to replicate their genome frequently, it may account for the extremely high number of the genomic copy of the symbiont and cell organelles. Both the slimming of the genome (Charles and Ishikawa, 1999) and its A/T richness (Ishikawa, 1987; Ohtaka and Ishikawa, 1993) will favor frequent replication of the genome.

From an evolutionary point of view, the high copy number of the *Buchnera* genome may explain how this bacterium has slowed down Muller's ratchet (Muller, 1964), which otherwise would bring about a serious problem to small and asexual populations such as that of *Buchnera* (Ohta, 1987; Moran, 1996). There is a possibility that multiple copies of the *Buchnera* genome are homologous, rather than exactly identical to each other. This, in turn, raises the possibility that different copies carry distinct alleles like homologous chromosomes of eukaryotes. Provided that recombination between these homologous copies takes place, it will effectively remove mildly deleterious mutations in a bundle that otherwise accumulate in the population of *Buchnera*. Thus, the presence of multiple genomic copies may enable *Buchnera*, asexual organisms, to enjoy mechanisms somewhat similar to sexuality to overcome deleterious mutations accumulating.

Recently, we estimated genomic copy number per *Buchnera* cell from host insects at various developmental stages by fluorimetry and real-time quantitative PCR (Komaki and Ishikawa, 2000). The results indicated that the genomic copy number increases during post-embryonic development of insects to adulthood, and that it decreases during host's aging. DAPI-staining indicated that the distribution of the genomic DNA in the *Buchnera* cells from old insects tends to aggregate, suggesting that intracellular structure of the genomic DNA of *Buchnera* varies in response to the physiological conditions of their host.

Gene Repertoire of *Buchnera*

Buchnera isolated from bacteriocyte are not culturable in any nutritional medium, which once hindered molecular approaches for their genes and gene expression. The development of PCR technique in the 1990s completely changed the situation. For example, Baumanns group in Davis, making the most of this technique using *E. coli* sequences as primers, identified and sequenced, more than 100 genes of *Buchnera* from the wheat green aphid, *Schizaphis graminum* (Baumann et al., 1997). Naturally, however, this approach alone did not supply information as to which genes have been lost from *Buchnera* as the result of intracellular symbiosis. With this in mind, we set about sequencing the *Buchnera* genome.

General features of Buchnera genes

The entire sequence of the genomic DNA of *Buchnera* from *A. pisum* was determined using the whole genome random shotgun sequencing method. It turned out that the genome is composed of one 640,681 kb chromosome and two circular plasmids, pLeu and pTrp (Shigenobu et al., 2000). The pLeu plasmid is 7,786 bp and has 7 ORFs including a *leuABCD* operon, and the pTrp plasmid has at least two tandem repeats of the *trpEG* operon. *Buchnera* contain a single copy of each of the three types of ribosomal RNA and 32 transfer RNA genes. We identified 583 ORFs, whose average size is 988 bp, occupying about 88% of the entire genomic sequence. This gene density in the genome is much the same as in the *E. coli* genome. Of interest is that the predicted isoelectric points (pIs) of these gene products are, on average, much more basic than those of polypeptides of other bacteria. This feature of *Buchnera* proteins has been already noted (Sato and Ishikawa, 1997; Matsumoto et al., 1999), and is mostly due to the increased occurrence of lysine in *Buchnera* polypeptides, which probably results from the A/T pressure imposed upon the *Buchnera* genome. It is interesting to know if the high pIs reflect the intracellular milieu where the bacterium lives.

Similarity search permitted the functional assignment of 500 of the 583 ORFs, and other 80 ORFs are homologous to hypothetical proteins deposited for other bacteria. Only three ORFs do not find their matches at present. This is a surprisingly small number, because in all the genomes, including those of parasitic bacteria, sequenced to date, at least, 20% of genes are unknown ones. Of the 583 ORFs, as many as 569 have orthologs in the *E. coli* genome. Moreover, in

Table 1. Comparison of gene numbers among several bacteria (Shigenobu *et al.*, 2000)

Function	Bu	Ec	Hi	Rp	Mg
Amino acid biosynthesis	55	131	71	6	0
Nucleotide metabolism	34	58	50	14	19
Energy metablism	51	243	143	67	33
Lipid metablism	6	48	34	25	8
Transport and binding	18	427	165	38	33
Regulation	7	178	64	14	5
Total genes	583	4,289	1,709	834	480

Bu, *Buchnera*; Ec, *Escherichia coli*; Hi, *Haemophillus influenzae*; Rp, *Rickettsia prowazekii*; Mg, *Mycoplasma genitalium*.

general, the most similar counterparts of *Buchnera* proteins are those of *E. coli*, and the gene orders in *E. coli* operons are well conserved in *Buchnera*. Based on these observations, we conclude the *Buchnera* genome is a subset of the *E. coli* genome.

Buchnera are similar to endocellular and epicellular parasites such as *Rickettsia prowazekii* (Andersson et al., 1998) and *M. genitalium* (Fraser et al., 1995) in that they have lost many genes that free-living bacteria share in common. However, the genes left behind in the genomes of these parasites and *Buchnera* are quite different. The parasitic bacteria depend on their hosts for most nutrients, and the reduction of their genome size is, at least partly, due to the loss of genes for biosynthesis of these nutrients. However, nutritional and physiological studies show that *Buchnera* are providers, rather than recipients, of biosynthetic products including the essential amino acids and vitamins, to their hosts (Sasaki and Ishikawa, 1995; Douglas, 1998; Nakabachi and Ishikawa, 1999). Such a difference of *Buchnera* is clearly reflected on their gene repertoire.

Genes for amino acid syntheses

The *Buchnera* genome contains as many as 54 genes for the synthesis of amino acids, which amount to almost 10% of its total genes (Table 1). This forms a striking contrast to gene repertoire of parasitic bacteria, which contain only a few, if any, genes in this category (Fraser et al., 1995; Andersson et al., 1998; Stephens et al., 1998). Whereas *E. coli* contain about twice as many as genes in this category, and are capable of biosynthesis of all necessary amino acids, *Buchnera* have selectively lost those genes for biosyntheses of the non-essential amino acids that animals can synthesize. Instead, unlike parasitic bacteria, *Buchnera* retain virtually all the genes for biosyntheses of essential amino acids that animals themselves cannot synthesize. This suggests that *Buchnera* provide the host with amino acids the host cannot synthesize, and that, conversely, the host provides *Buchnera* with what these bacteria cannot synthesize. In addition, as precursors of some essential amino acids are non-essential amino acids, such as glutamate and aspartate, the biosynthetic pathways of the host and symbiont are not only complementary, but also mutually dependent. This gene repertoire concerning amino acid syntheses is consistent with experimental evidence that aphids recycle the amino group of nitrogenous waste products as glutamine, which *Buchnera* use as a substrate for the synthesis of the essential amino acids (see section 4; Sasaki and Ishikawa, 1995; Douglas, 1998).

Mutual dependence with the host as seen in gene sets

A similar mutual dependence between the host and symbiont is predicted for biosynthesis of coenzyme A (CoA). Judging from the gene repertoire, *Buchnera* can synthesize pantothenate from pyruvate, whereas they do not contain genes for the pathway from pantothenate to CoA. Conversely, as generally known, animals are able to produce CoA from pantothenate, but not pantothenate from pyruvate. Despite a dramatic reduction of the gene number, *Buchnera* retain complete gene sets for the sulfur reduction pathway and biosynthesis of cysteine. The host must benefit much from these ablities of the symbiont, because insects, in general, cannot reduce sulfate to sulfide. Indeed, experimental evidence suggests that the *Buchnera*-bacteriocyte system is responsible for sulfate assimilation in aphids (Douglas, 1988). In contrast to obligatory parasitic bacteria whose genomes have been sequenced to date, *Buchnera* retain almost complete sets for biosyntheses of nucleotides. However, it is yet to know whether the synthetic pathways are for the host or for their own use.

Genes for energy metabolism

The genome data clearly indicate that *Buchnera* are aerobic bacteria. This seems reasonable as these bacteria inhabit the bacteriocyte that receives an ample supply of oxygen through the tracheole and contains many mitochondria in the cytoplasm. *Buchnera* have complete gene sets for the glycolytic pathway, the pentose phosphate cycle, and aerobic respiration. In the *Buchnera* genome, the NADH dehydrogenase operon and the cytochrome *o* operon are conserved with the same gene arrangement as in the *E. coli* genome. In addition, *Buchnera* have an FOF1 type ATP synthase operon, suggesting that these bacteria are able to produce ATP, using the proton electrochemical gradient generated by electron transfer system (ETS). In contrast, *Buchnera* lack genes responsible for fermentation and anaerobic respiration. With all these results indicating that these bacteria respire aerobically, to our surprise, *Buchnera* do not have a gene set for the citric acid cycle, except genes for the 2-oxogutarate dehydrogenase complex.

This raises an interesting question: How do *Buchnera* respire aerobically without operating the citric acid cycle? One possibility is that their glycolytic pathway supplies an amount of NADH enough to

operate ETS. In considering that *Buchnera* do not have a pathway to oxidize NADH through fermentation, and that phloem sap, the diet of aphids, is rich in sugar, this is a plausible explanation. It is also possible that *Buchnera* somehow import electron donors indirectly from the surrounding cytoplasm of bacteriocyte or mitochondria in the vicinity. Two other possibilities assume that *Buchnera* do have the citric acid cycle in spite of the lack of its gene set. One possiblity is importation of relevant enzymes from the host cytoplasm exactly as do mitochondria. Alternatively, *Buchnera* may use multifunctional proteins, in place of the authentic enzymes, to operate the cycle. This may not be a far-fetched explanation as molecular evolution of *Buchnera* is very unique (Shigenobu et al., 2001; see in section 7). In addition, there is an example in which a *Buchnera* homolog of an *E. coli* protein has evolutionarily acquired, at least, one more activity in addition to the original function (see in section 6).

Essential genes missing from Buchnera

In contrast to relative richness in genes for the biosyntheses of nutrients, *Buchnera* have lost many genes that seem to be essential to maintain their identity as independent cells. *Buchnera* lack many transporter genes that are shared in common by all bacterial species sequenced to date. This finding is surprising because intracellular symbioses, in general, should necessarily be based on frequent exchanges of various substances between the two associants. For example, *Buchnera* have only a few genes for the ABC transport system, which is the major class of cellular translocation machinery with many paralogous genes. *Buchnera* retain genes for phosphoenolpyruvate-carbohydrate phosphotransferase systems (PTSs) for glucose and mannitol. Apart from these transporters, no other substrate-specific transporter genes are present in the *Buchnera* genome.

Genes for various regulatory systems are almost completely missing from the *Buchnera* genome. Among these are the genes for two-component regulatory systems that generally control gene expression in response to environmental changes. This is possible because the intracellular conditions are stable for the life of *Buchnera*. It is particularly noteworthy that no transcriptional regulator of amino acid biosyntheses is present in *Buchnera*. Consequently, it is probable that, so far as substrates are available, *Buchnera* produce the essential amino acids as much as the host demands. In general, *Buchnera* genes do not have leader sequences. Also, *Buchnera* are not equipped with transcriptional attenuation systems. It is possible that gene expression of *Buchnera* is not controlled by itself, but by the symbiotic system as a whole.

Buchnera have only a few genes for cell surface components such as lipoproteins. The gene repertoire shows that the cell surface of *Buchnera* is structurally vulnerable. This is in contrast to parasitic bacteria, which generally have complex and flexible surface structures, probably to evade attack by the host immune system and to survive harsh environments. The structural fragility of *Buchnera* may be caused by its prolonged intracellular life, sheltered from attack by the host and foreign enemies. In view of the gene repertoire, *Buchnera* have a limited capacity for DNA repair and recombination. It is striking that the *recA* gene is missing from the *Buchnera* genome, as RecA is the most crucial component for the homologous recombination reaction. Similarly, in the *uvr* excision repair system, *Buchnera* lack *ubrABC*. All sequenced eubacterial genomes except for *Buchnera* retain *recA* and *uvr ABC* genes (Eisen and Hanawalt, 1999). This implies that the repair system and the recombination mechanism of this symbiotic bacterium are severely impaired. The absence of a series of gene responsible for the SOS system, *recA*, *lexA*, *umuCD* and *uvrABC*, also indicates that the *Buchnera* genome is vulnerable to DNA damage. Genes for DNA methylation and restriction are also missing, providing further evidence that *Buchnera* have limited defence. It is possible that the high copy number of the genome permits *Buchnera* to eliminate damaged DNA, rather than repair it (Komaki and Ishikawa, 1999).

Of further interest is that many genes are missing from the *Buchnera* genome, though their direct or indirect products are obviously present in the *Buchnera* cell. One typical example is the genes for phospholipid biosynthesis. Although phospholipid is an indispensable component in the formation of the membrane lipid bilayer, genes necessary for its biosynthesis are completely missing from *Buchnera*. Possibly, this symbiont either imports phospholipid from the host or synthesizes it, employing relevant enzymes transferred from the host cell, exactly as do mitochondria and plastids.

Symbionin, a GroEL Homolog

In the course of studies on the host-symbiont interactions in the bacteriocyte of the pea aphid, *A. pisum*, it was demonstrated that *Buchnera*, when housed in the bacteriocyte, selectively synthesize a particular protein in a large amount. This protein, which we designated "symbionin", is supposed to play a key role in the aphid-*Buchnera* symbiosis (Ishikawa, 1982; 1984).

Symbionin as a molecular chaperone

Symbionin was shown to be a 14 subunit homooligomer of 63 kDa, and to be composed of two stacked rings of seven subunits each (Hara and Ishikawa, 1990). This native structure, partial amino acid sequences determined by Edman degradation (K. Kakeda, unpublished data), and the result of immunological analysis using anti-symbionin antiserum revealed that symbionin is very similar to GroEL (Hara et al., 1990), a stress

protein of *E. coli* that is a member of the chaperonin family of molecular chaperone (Hemmingsen et al., 1988). Indeed, nucleotide sequence determination of the symbionin gene revealed that this protein is 85.8% identical with GroEL of *E. coli* at the amino acid sequence level (Ohtaka et al., 1992). In addition, it was demonstrated that symbionin, in place of GroEL, chaperones polypeptides, including itself, both *in vivo* (Ohtaka et al., 1992) and *in vitro* (Kakeda and Ishikawa, 1991; Morioka and Ishikawa, 1992). It is yet to know how the chaperone activity of symbionin contributes to this symbiotic system. One possibility is that symbionin chaperones polypeptides imported from the cytoplasm of bacteriocytes. Since many genes are missing from *Buchnera* (Shigenobu et al., 2000), these bacteria may have to import substitutes for these gene products from bacteriocytes (see section 5). It can be symbionin that chaperones these imports.

Symbionin as a histidine protein kinase

When *Buchnera* cells isolated from bacteriocytes are subjected to heat shock, they synthesize a phosphorylated form of symbionin in addition to several other stress proteins (Morioka and Ishikawa, 1992). During studies on the mechanisms of phosphorylation of symbionin by heat shock, it was found that symbionin, when incubated with ATP at elevated temperatures, is phosphorylated *in vitro* autocatalytically in an energy-coupling manner. The phosphoryl group transferred to symbionin, in turn, is transferred to other molecules including ADP. We also showed that symbionin catalyzes the transfer of phosphoryl group from ATP to GDP (Morioka et al., 1993). These results suggest that symbionin functions dually, serving as an intermediary substrate on one hand, and as an enzyme catalyzing the transfer of phosphoryl group on the other hand. This molecular property of symbionin is reminiscent of histidine protein kinases of two-component regulatory systems that regulate various adaptive responses in both prokaryotes and eukaryotes through phosphoryl group transfer (Ohta et al., 1992; Parkinson and Kofoid, 1992; Chang et al., 1993).

In an effort to figure out biological significance of the transfer of phosphoryl group in *Buchnera* symbiont, we looked for proteins that receive phosphoryl group from symbionin. Proteins were extracted from isolated *Buchnera* cells and resolved electrophoretically. When the proteins were blotted onto a membrane and incubated with ^{32}P-labeled symbionin, the radioactivity was found on several proteins, indicating that the phosphoryl group was transferred from symbionin to multiple proteins of *Buchnera*, which include an OmpF homolog and a histone H1 homolog (Morioka et al., 1994; Morioka and Ishikawa, 1998). Genome analysis demonstrated that the histone H1 homolog is not encoded by the *Buchnera* genome, and is one of the proteins that are imported from the cytoplasm of bacteriocytes (Shigenobu et al., 2000).

```
                                133
                    Ala Val Glu Glu Leu Lys Ala Leu Ser Val Pro Cys Ser
GroEL               TGCAGTTGAAGAACTGAAAGCGCTGTCCGTACCATGCTCT
Symionin            G
                    TGCTGTAGAAGAATTAAAACATTTATCTGTACCATGTTCTG
                    Ala Val Glu Glu Leu Lys His Leu Ser Val Pro Cys Ser
```

Fig. 4. Comparison between GroEL and symbionin around the codon 133. An amino acid replacement between Ala-133 and His-133 resulted from three consecutive nucleotide replacements at the codon 133 (Ohtaka et al., 1992).

Peptide sequence and thin-layer chromatographic analysis of the ^{32}P-labeled tryptic fragments of the phosphorylated symbionin revealed that the site of phosphorylation is His-133 (Morioka et al., 1994). This, taken together with the results mentioned above, suggests that in function symbionin mimics histidine protein kinases, or sensor molecules, of two-component regulatory systems. However, symbionin is not similar to any known histidine protein kinase in amino acid sequence.

Evolution of GroEL homolog

The phosphotransferase activity is unique to symbionin, and the activity is not found in GroEL of *E. coli* that is more than 85% identical with symbionin in amino acid sequence. It is conceivable that symbionin, a GroEL homolog, has acquired this unique activity through adaptation to the intracellular environment in the course of evolution. The lack of genes for two-component regulatory systems in the *Buchnera* genome (Shigenobu et al., 2000) and the functional similarity of symbionin to histidine protein kinases raise a possibility that in *Buchnera* symbionin plays a central role in signal transduction by relaying phosphoryl group.

His-133 is the phosphorylation site of symbionin, and the corresponding site of GroEL is occupied by Ala that is by no means phosphorylated (Ohtaka et al., 1992). This suggests that amino acid substitution at this position endowed symbionin with the phosphotransferase activity (Morioka et al., 1994). In this regard, it should be emphasized that the histidine codon at the position 133 of symbionin has been created as a result of three consecutive nucleotide substitutions between *groEL* and the symbionin gene (Fig. 4). In considering that nearly all the other nucleotide substitutions between the two genes are synonymous or analogous, such non-neutral consecutive substitution is a very rare event (Ohtaka and Ishikawa, 1993).

To further assess a unique amino acid substitution at the position 133 of symbionin, we compared nucleotide sequences of symbionins of *Buchnera* species from three closely interrelated aphids, *A. pisum*, *A. kondoi* and *A. solani* (Komaki et al., 1996). It turned out that, as expected from the close relationship of these host insects, predicted amino acid sequences of the three symbionins are more than 99% identical with one another. GroEL was about 86% identical with each symbionin. The three symbionin genes were 92-94%

identical with one another in nucleotide sequence. Any one of the three symbionins was different from the other two at only five positions out of the total 548 amino acid residues. One of the five positions was the position 133 where, in *A. kondoi* and *A. solani*, Asn had been substituted for His in *A. pisum*. Interesting enough, Asn is an amino acid that is also subject to phosphorylation to give rise to an unstable product.

In this context, it is noteworthy that many GroEL homologs, both bacterial and organellar, examined to date contain Lys at the position 133 (Gupta, 1990). The amino acid is also phosphorylated to form the phosphoramidate bond. As both phosphoasparagine and phospholysine, just as phosphohistidine, have high standard free energy of hydrolysis, it is likely that these GroEL homologs also have the energy-coupling phosphotransferase activity, though not evidenced yet. It is probable that the codon 133 of GroEL homolog genes is neutral to the chaperone activity, and a hot spot of molecular evolution. Thus, taking advantage of some base substitutions at this codon, GroEL homologs may be in the process of evolutionary change from a mere chaperone to a multifunctional protein (Komaki et al., 1996).

According to a Dutch group, symbionin is synthesized by *Buchnera*, then secreted into the hosts hemolymph, and serves there as a carrier of plant viruses whose vectors are aphids (van den Heuvel et al., 1994; Morin et al., 1999). This plausibly explains why symbionin is produced in such a large amount. However, no other groups have confirmed their observation that symbionin molecules are secreted out of the *Buchnera* cell.

Recently, Yoshida et al. (2001) characterized a toxic substance produced by a pit-building antlion that paralyzes its preys. According to their report, the toxin is a GroEL homolog that is synthesized by symbiotic bacteria inhabiting the saliva of the insect. Only 11 amino acid residues in the GroEL homolog have alignments different from the residues in GroEL of *E. coli*. All these substitutions seem to be neutral in terms of the chaperone activity of the GroEL homolog. They pointed out that some of the substitutions directly conferred toxicity on the GroEL homolog. This suggests that GroEL homologs contain some other positions, in addition to the position 133, that are susceptible to mutation to lead to a multifunctional protein. It is intriguing that a few of the amino acid substitutions responsible for the toxicity are shared in common by symbionin.

Species-Specific Evolution of *Buchnera* Genes

Buchnera species have been locked inside their hosts for an evolutionarily long time, with a small population size and substantially no recombination. As a result, their genomes are expected to have accumulated mildly deleterious mutations in a process referred to as Mullers ratchet (Moran, 1996; Andersson and Kurland, 1998). Indeed, it was reported that the genes of *Buchnera* exhibit unusually low ratios of synonymous-to-nonsynonymous substitutions, as compared with those for the corresponding genes of enterobacteria (Clark et al., 1999; Wernegreen and Moran, 1999). This accumulation of nonsynonymous substitutions is compatible with the hypothesis of increased fixations of deleterious mutations, whose consequence will be the inactivation and eventual deletion of nonessential genes (Moran, 1996). It is apparently consistent with this reductive evolution that *Buchnera* is the smallest bacterium in both genome size and number of genes among the bacteria sequenced to date except for *M. genitalium* (Charles and Ishikawa, 1999).

Non-random distribution of mutations

Recently, we compared all the ORFs of *Buchnera* with those of 34 other prokaryotic organisms, and estimated the effect of the accelerated evolution of this bacterium on the functions of its proteins (Shigenobu et al., 2001). It was revealed that *Buchnera* proteins contain many mutations at the sites where sequences are conserved in their orthologs in many other organisms. In addition, amino acid replacements at the conserved sites most frequently resulted in the formation of physicochemically distinct amino acids. These results suggest that conformations and functions of *Buchnera* proteins have been seriously impaired or strongly modified. Indeed, extensive loss of functional motifs was observed in some *Buchnera* proteins. In many *Buchnera* proteins mutations were not detected evenly throughout an entire molecule, but tended to accumulate in some functional units, which possibly led to loss of specific functions. As *Buchnera* has an unusual and limited gene repertoire, it is conceivable that the manner of interactions among its proteins has been changed, and thus functional constraints over their amino acid residues have also been changed during evolution. This may account for the loss of some functional units only in the *Buchnera* proteins. We obtained sound evidence that amino acid replacements in *Buchnera* are not always deleterious, but neutral or, in some cases, even positively selected. One typical example of this tendency is observed in the evolution of the GroEL homolog, as mentioned in the previous section.

Mechanisms of Buchnera-specific evolution

As mentioned already, the *Buchnera* genome seems to be under the influence of a strong AT pressure (Ishikawa, 1987; Sueoka, 1988). We examined whether the increased AT content of codons accounts for the amino acid exchange patterns observed in *Buchnera*. In doing so, we focused on the first and second

positions of the codons, because nucleotide substitutions at these positions contribute to most amino acid replacements. As a result, it was shown that the tendency of amino acid replacements is strongly influenced by the increase in AT in codons: mutations with increasing AT amounted to 72%; those with decreasing AT, only 9%. This suggests that the genome of the last common ancestor (LCA) of *Buchnera* and *E. coli* was, at least in ORFs, richer in GC than that of *Buchnera*. This further suggests that a large part of the amino acid replacements in *Buchnera* proteins resulted in an increase in amino acids encoded by high AT codons. Taking into account the higher AT content in intergenic regions than that in ORFs in *Buchnera*, it is concluded that the major driving force of the biased mutations in *Buchnera* proteins includes the AT pressure imposed on the *Buchnera* genome.

Relationship between *Buchnera* and Hosts

Vertical transmission

Most aphid species reproduce parthenogenetically throughout the greater part of a year. In their parthenogenesis, they transmit *Buchnera* cells to the next generation by pushing them out of bacteriocytes into early embryos developing in the ovariole. Recently, Miura and his colleagues succeeded in observing the detailed process of transmission of *Buchnera* during parthenogenesis of the pea aphid, *A. pisum*. According to their observations, transmission of *Buchnera* is an event at the developmental stage of blastula in which differentiation of the germ line is nearly completed. The blastula is surrounded by follicular epithelium, whose cell layer is thicker in the posterior region. This region takes up a bacteriocyte from the mother, which represents the initiation of transmission. Cell membrane of the bacteriocyte incorporated by the follicle and envelope of the embryo are temporarily fused together to form a channel, through which a mass of *Buchnera* cells flow into the blastocoel. The blastocoel at this stage has been divided into the anterior and posterior portions by the germ line cells that flowed into the space previouly. It is the posterior portion that incorporates the mass of *Buchnera* cells. Since immediately after this incorporation, invagination of the archenteron starts also at the posterior portion, it seems as if the archenteron pushes in the *Buchnera* mass to the blastocoel. The blastocoel at this stage is multinuclear, and each nucleus in the posterior portion and numerous *Buchnera* cells together are subjected to cellularization, which apprently gives rise to the bacteriocyte of the next generation (Fig. 5). It should be emphasized that the entry of *Buchnera* cells into embryo is not an accidental event, but a precisely programed process of embryogenesis of aphids (T. Miura, C. Braendle, S. Kombhampahti and D. Stern, unpublished observations). This probably reflects a long history of this symbiosis and the strong dependence of aphids on *Buchnera*.

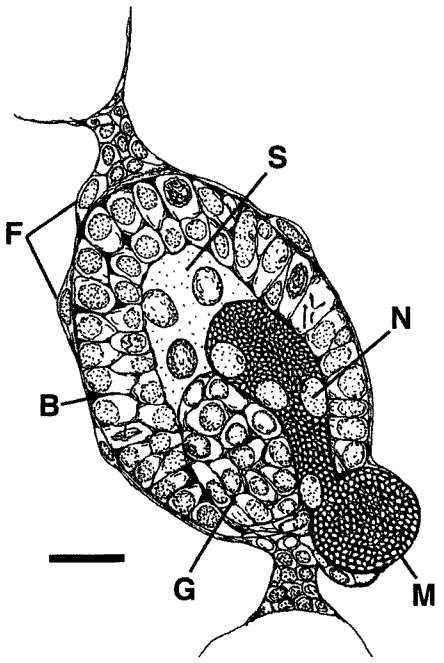

Fig. 5. A parthenogenetic blastula of the pea aphid, *Acgrthosiphon pisum* (figure drawn by T. Miura). B, blastoderm; F, follicle cells; G, germ line cells; M, mass of *Buchnera* cells from mother; N, nucleus of a prospective bacteriocyte; S, blastocoel. scale bar = 10 μm.

Holocyclic aphids reproduce bisexually once a year. In this case, sexual females relay *Buchnera* cells to the next generation by inserting them into the yolk in the course of oogenesis (Ishikawa et al., 1986). As is often the case with intracellular symbionts, *Buchnera* are never transmitted by way of spermatozoa. Consequently, *Buchnera* cells that enter the male cannot but come to a dead-end with no means to propagate their progeny. Sociobiologically, such a situation will tend to evolve a mechanism that prevents *Buchnera* from being transmitted to the male embryo. Indeed, *Buchnera* are not detected in males in some aphid species (Buchner, 1965). In this context, the same situation is expected in the soldier caste, which does not produce progeny, of eusocial aphids (Aoki, 1977). In reality, whether soldiers contain *Buchnera* or not depends upon aphid species (Fukatsu and Ishikawa, 1992). Males and soldiers of some aphid species will feed, whereas those of others will not. Non-reproductive individuals that feed probably need *Buchnera* to supplement the diets. Thus, it is likely that the presence of *Buchnera* in males or soldiers is a reflection of the hosts interest rather than that of *Buchnera* the symbiont.

Buchnera and hosts development

It seems that the number of bactriocytes in an individual aphid does not increase appreciably throughout its embryonic and post-embryonic development.

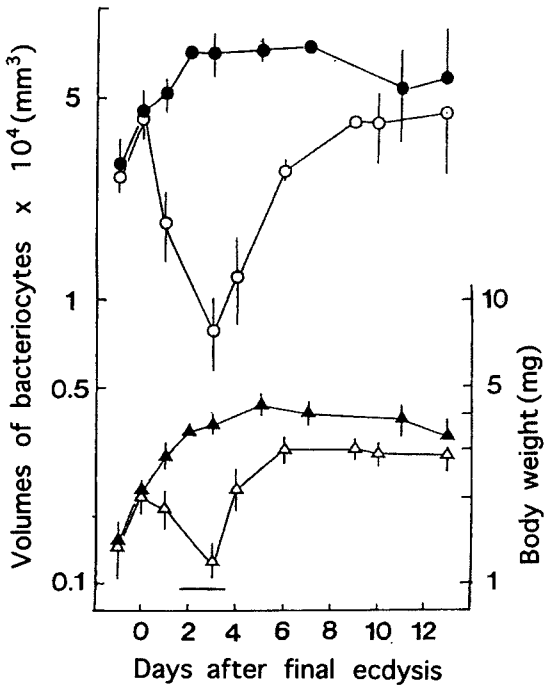

Fig. 6. Changes in volume of bacteriocytes (○, ●) and fresh body weight (△, ▲). Each value was expressed as mean±SD. Closed symbols, apterae, n=10 to 25; open symbols, alatae, n=10 to 56. Horizontal bar designates the flying period of alatae (Hongoh and Ishikawa, 1994).

Instead, the size of these cells markedly increases as the insect grows. It has long been known that the ploidy of bacteriocyte is generally high and sometimes attains to as high as 512n in adult aphids (Buchner, 1965). This suggests that DNA replication has taken place repeatedly in the cell without accompanying cell division. The fact that bacteriocytes harbor *Buchnera* cells incessantly at a high density suggests that the increase of *Buchnera* cells in the bacteriocyte is well coordinated with the size increase of bacteriocyte. It is conceivable that *Buchnera* reduces the frequency of its cell division to coordinate with a slow increase of the size of bacteriocytes, which may, at least in part, account for the high copy number of genome in each *Buchnera* cell. The result that the copy number of the *Buchnera* genome tends to increase during post-embryonic development of the host insect also suggests the presence of such coordination (see section 4). In this regard, it is of interest to know of what is the genomic copy number of *Buchnera* in a freshly-formed bacteriocyte of early embryos. However, an answer to this question will not be easily attainable because of technical difficulties in manipulating early embryos of a small insect.

Buchnera as nutrient reserves

In general, aphids are known for their polymorphism. Even parthenogenetic females exhibit two morphs, apterae or wingless forms and alatae or winged forms. Unlike apterae, alatae bear, a special mission of migratory flight in addition to larviposition,. They are driven to flight a few days after the final ecdysis in order to settle on new host plants and create new colonies (Kring, 1972). During this period, alatae undergo striking changes, both morphological and physiological, including development and degeneration of flight muscles, the changes that are never observed in apterae. We studied the changes of bacteriocytes of alatae during this period in comparison with those of apterae at the same stage (Kobayashi and Ishikawa, 1993; 1994; Hongoh and Ishikawa, 1994). As a result, it was revealed that the total volume of bacteriocytes per aphid changes quite differently between the two morphs after the final ecdysis. While, in apterae, the total volume increases until the onset of larviposition, and gradually decreases thereafter, in alatae it decreased sharply over the first three days subsequent to the final ecdysis (Hongoh and Ishikawa, 1994). This period exactly coincides with the period in which alatae develop their flight muscles to set out for migratory flight. Throughout this period, alatae decrease their body weight, and the protein content of their indirect flight muscles increases in inverse proportion to their body weight. Immediately after settling on new host plants, alatae begin feeding, which stimulates the degeneration of flight muscles on one hand, and the increase of the body weight on the other hand, followed by the initiation of larviposition (Kobayashi and Ishikawa, 1993). The beginning of feeding also stimulates a rapid restoration of the total volume of bacteriocytes (Fig. 6; Hongoh and Ishikawa, 1994).

Over the first few days subsequent to the final ecdysis, alatiform aphids, unlike apterae, do not ingest an appreciable amount of food (Mittler, 1958; Kobayashi and Ishikawa, 1993), which suggests that their flight muscles have to develop without supply of nutrients by feeding. The rise and fall of the total volume of bacteriocytes during the period before and after migratory flight are in exactly inverse relation to those of the protein content of indirect flight muscles. This suggests that a possible source of nutrients for the developing muscles is bacteriocytes containing *Buchnera*. Our observation that not only the total volume of bacteriocytes but also the density of *Buchnera* cells in bacteriocytes decreases in this period suggests that *Buchnera* cells, rather than the bacteriocyte itself, serve as a major nutrient source.

The possibility that *Buchnera* serve as nutrient reserves for the host was also sustained by the experiments in which aphids were kept starved. As far as aphids, irrespective of morph, were kept starved, their total bacteriocyte volume was consistently decreased at a much higher rate than that of the decline of their body weight, suggesting that under the nutritionally adverse conditions aphids will reduce their

endosymbiotic system desidedly in order to preserve their own life at the cost of *Buchnera*. This situation was most typically demonstrated with alatae at the teneral period. Starving them, on one hand, induced a sharp decline of their total bacteriocyte volume, and, on the other hand, prevented their indirect flight muscles from breaking down (Kobayashi and Ishikawa, 1993). When the nutritional conditions were improved by resumption of the feeding, the total volume of bacteriocytes immediately restored the original value, suggesting that the change is reversible in nature. Thus, our conclusion is that aphids will survive the nutritionally adverse conditions consuming their endosymbionts as nutitional reserves. For the present, it is merely a matter of conjecture whether or not, under the adverse conditions, aphids reduce the total symbiosis including that of their developing embryos.

Concluding Remarks

Many eukaryotic cells constitute the sole habitat for a vast and varied array of prokaryotic lineages (Buchner, 1965). These intracellular associations have evolved repeatedly and have had major consequences for the diversification of both bacteria and host. The magnitude of these consequences is immediately evident if one considers the examples of mitochondria and plastids, which are now widely acknowledged to be descended from prokaryotes that invaded intracellular habitats (Margulis, 1970; Gray et al., 1999). One of the ongoing examples that demonstrate major consequences of these associations is seen in the combination of insects and bacteria. The great diversity of insects is, at least partly, due to their frequent associations with mutualistic endosymbionts, such as *Buchnera*, which allow hosts to exploit niches that would otherwise be nutritinally unsuitable. Further molecular studies on these associations, combined with detailed biological investigations, promise to elucidate still unknown aspects of organismal evolution.

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