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CHAPTER 2.3

Bacteriocyte-Associated Endosymbionts of Insects

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Introduction

Intracellular associations between bacteria and insects are widespread in nature (Baumann and Moran, 1997; Buchner, 1965; Dasch et al., 1984; Douglas, 1989; Houk and Griffiths, 1980). Extensive studies of the natural history of such associations have led to the conclusion that they are commonly found in insects that utilize diets containing an excess of one class of compounds but a deficiency of some essential nutrients (Buchner, 1965; Dadd, 1985). It was thought that the function of the endosymbionts was to rectify this imbalance by the synthesis of these essential nutrients for the host. Extensive compilations of the occurrence of endosymbionts in different groups of insects are found in Buchner (1965) and Dasch et al. (1984). Because most of the prokaryotes involved in such associations are not cultivable on common laboratory media, their characterization had to await the development of recombinant DNA methodology. The past 10 years have witnessed the initiation of studies on the intracellular association of prokaryotes with a variety of insect hosts. In this chapter we will provide an overview of the evolution, and where possible, genetics and physiology of such recently studied associations. A summary of some of their features is presented in Table 1, and the phylogeny of the endosymbionts based on 16S rDNA is presented in Fig. 1.

The diversity of symbiotic associations and problems of definitions have been previously discussed and will not be considered here (Smith and Douglas, 1987; Werren and O'Neill, 1997). Some of the phylogenetic studies have included few host taxa and are thus not entirely conclusive; nevertheless, current results suggest that most of the associations considered in this chapter have common features and represent a relatively well-defined type. To aid presentation we will describe these common features, which are established from recent, largely molecular, studies as well as from older investigations based on morphological analyses. References to the earlier studies are found in Buchner (1965), who arrived at similar conclusions. References to recent studies are given as each association is considered.

The associations listed in Table 1 and Fig. 1 are the results of infections of various insect lineages with different prokaryotes. These associations became stable, resulting in the emergence of a new composite (of host and endosymbiont) organism. The endosymbiont became heritable through the acquisition of mechanisms ensuring vertical, maternal transmission to progeny. The association also became obligate, or beneficial, for host growth. Because the host depended on the association, and because horizontal or infectious transmission between hosts did not occur, the phylogeny of the endosymbionts is congruent with the phylogeny of the hosts. With some exceptions, heritable associations tend to become mutualistic (Lipsitch et al., 1995; Werren and O'Neill, 1997). In most cases, the host cannot survive without the endosymbiont, or the elimination of the endosymbiont has a deleterious effect. Although the advantage for the host is in most cases apparent, the advantage for the endosymbiont is not always clear. Perhaps it is more correct to think that the host domesticates the endosymbiont for its own welfare, utilizing functions that are present in the prokaryote but lacking in the host (Douglas and Smith, 1989; Maynard Smith and Szathmáry, 1995). In this chapter, the organism which is present in all the species of an insect group and which appears to be of essential value to the host is designated by either its scientific name or, if one is lacking, by the term primary (P-) endosymbiont.

Superimposed on this fundamental association may be associations with additional endosymbionts. Although these are heritable, they appear to be the result of multiple independent infections, horizontal transmission, or both. Since these endosymbionts may be absent in some hosts, their contribution to the welfare of the organism may not be major or essential. These organisms are designated as secondary (S-) endosymbionts. In this connection it is relevant that some bacterial strains may exist within the body cavity of insects for long periods without obvious deleterious effects, thus serving as

Table 1. General properties of the considered endosymbiotic associations.[a]

Host category[b]	Principal host food source	Symbiont designation	16S rRNA group or other taxonomic desgnation
Order: Homoptera Suborder: Sternorrhyncha Superfamily: Aphidoidea			
Aphids	Phloem sap	*Buchnera aphidicola* S-endosymbiont	γ-*Proteobacteria* *Enterobacteriaceae*[c]
Superfamily: Psylloidea			
Psyllids	Phloem sap	P-endosymbiont S-endosymbiont	γ-*Proteobacteria* γ-*Proteobacteria*
Superfamily: Aleyrodoidea			
Whiteflies	Phloem sap	P-endosymbiont S-endosymbiont	γ-*Proteobacteria* *Enterobacteriaceae*
Superfamily: Coccoidea Family: Pseudococcidae			
Mealybugs	Phloem sap	P-endosymbiont	β-*Proteobacteria*
Order: Diptera Family: Muscidae Genus: *Glossina*			
Tsetse flies	Vertebrate blood	*Wigglesworthia glossinidia* (P-endosymbiont) *Sodalis glossinidius* (S-endosymbiont)	γ-*Proteobacteria* *Enterobacteriaceae*
Order: Coleoptera Family: Curculionidae Genus: *Sttophilus*			
Weevils	Stored grain	P-endosymbiont	*Enterobacteriaceae*
Order: Hymenoptera Family: Formicidae Genus: *Camponotus*			
Carpenter ants	Plantnectar, honeydew, detritus and other sources	P-endosymbiont	γ-*Proteobacteria*
Order: Orthoptera Superfamily: Blattoidea			
Cockroaches	Universalists	*Blattabacterium cuenoti*	Flavobacterium-Bacteroides group
Order: Isoptera Family: Mastotermitidae Genus: *Mastotermes*			
Termites	Dead wood	*Blattabacterium cuenoti*	Flavobacterium-Bacteroides group

[a]See text for references.
[b]Taxonomy of the host according to Borror et al. (1989).
[c]As defined by Brenner (1984).

potential endosymbiont precursors of endosymbiotic associations (Boman and Hultmark, 1987; Faye, 1978).

These conclusions are tentative and, with the possible exception of the *Sitophilus*-endosymbiont association, are probably applicable to most of the endosymbiotic associations considered in this chapter. One well-studied, contrasting association is between many arthropods and the intracellular prokaryote, *Wolbachia* (O'Neill et al., 1997). Although this organism is typically heritable, being transmitted maternally, results of phylogenetic studies imply some incidence of horizontal exchange between very different lineages. *Wolbachia* causes a number of different reproductive alterations favoring the spread of infected host lineages and has properties characteristic of a parasite (O'Neill et al., 1997). Recent work may necessitate a modification of this conclusion because newly discovered *Wolbachia* in filarial worms appear to be essential for host survival and may show phylogenetic congruence with their hosts, indicating vertical evolution (Bandi et al., 1998, 1999).

Symbionts of Insects Which Utilize Plant Sap as Food

Aphids, psyllids, whiteflies and mealybugs share a number of common structural and nutritional

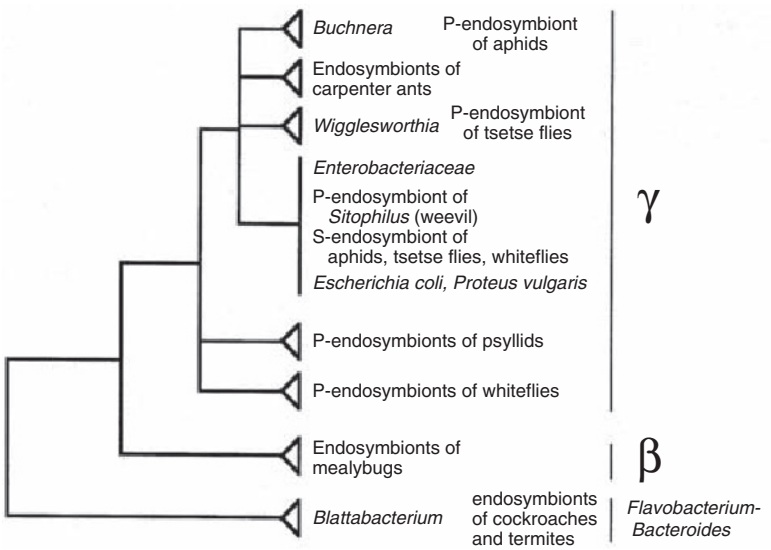

Fig. 1. Phylogenetic tree resulting from parsimony analysis of insect endosymbionts based on 16S rDNA sequence analysis. P-, primary endosymbiont; S, secondary endosymbiont; Greek letters, subdivisions of the Proteobacteria. References given in text.

properties (Borror et al., 1989) and constitute four separate lineages within the suborder Sternorrhyncha (order Homoptera; Campbell et al., 1994; von Dohlen and Moran, 1995). All of these groups feed predominantly or exclusively on plant phloem sap. This mode of life necessitates the penetration of plant tissue by flexible tubular mouthparts (stylets) and the ingestion of plant phloem sap. This diet is unbalanced, as it is rich in carbohydrates but deficient in amino acids and other nitrogenous compounds (Dadd, 1985; Sandström and Pettersson, 1994). Because of the low concentrations of nitrogenous compounds, phloem-feeding insects ingest a large amount of plant sap and then excrete the excess sugar as honeydew. This mode of feeding is conducive to the transmission of plant viruses, and members of the Homoptera are important vectors of viral plant pathogens (Blackman and Eastop, 1984; Gray and Banerjee, 1999; Sylvester, 1985). In addition, these insect populations may reach enormous numbers on plants, causing nutrient deprivation, leaf curling, and gall formation (Borror et al., 1989).

In spite of these common properties, aphids, whiteflies, psyllids, and mealybugs have different prokaryotic P-endosymbionts (Table 1, Fig. 1). These insects, like other animals, require ten essential amino acids, and endosymbionts are thought to upgrade the diet by synthesizing these essential amino acids and providing them to the host (Baumann et al., 1995, 1997a, b; Douglas, 1989; Moran and Telang, 1998). Of these four symbiotic associations, the most extensively studied is that between *Buchnera* (the P-endosymbiont) and aphids. This association will be considered in some detail and followed by a brief discussion of three other associations.

Aphid Endosymbionts

Buchnera—the Primary Endosymbiont of Aphids

Phylogeny

The initial characterization of the 16S-rDNA gene of *Buchnera* involved the use of an *Escherichia coli* 16S rDNA hybridization probe to perform a restriction enzyme and Southern blot analysis on total *Acyrthosiphon pisum* DNA, which established that this gene was present as a single copy (Unterman et al., 1989). Subsequently three overlapping DNA fragments were cloned and the 16S-rDNA gene was sequenced. In addition, bacteriomes were dissected from the aphid and the DNA purified. Restriction enzyme and Southern blot analysis gave the same results with whole aphid DNA and DNA obtained from dissected bacteriomes, indicating that the bacteriomes were the source of endosymbiont DNA. In all subsequent studies, the 16S rDNA was obtained by PCR amplification using whole aphid DNA preparations cloned into plasmid vectors and then sequenced (Munson et al., 1991b).

Based on 16S rDNA analysis, *Buchnera* is a distinct lineage within the γ-3 subgroup of the Proteobacteria (Fig. 1; Moran et al., 1993; Munson et al., 1991b; Unterman et al., 1989; van Ham et al., 1997). The closest known organisms are the endosymbionts of carpenter ants, endosymbionts of tsetse flies (*Wigglesworthia*), and members of the Enterobacteriaceae as defined by Brenner (Brenner, 1984; Aksoy, 1995a, b; Schröder et al., 1996). Phylogenetic analyses based on 16S rDNA indicate that these organisms are four separate lineages but do not permit

firm conclusions regarding their relationships to one another. *Buchnera* contains a single copy of rRNA genes which are arranged as two transcription units, 16S rRNA and tRNAGlu-23S rRNA-5S rRNA (Munson et al., 1993; Rouhbakhsh and Baumann, 1995). This organization of the rRNA genes into two transcription units is somewhat rare but also has been found in *Wolbachia* (Bensaadi-Merchermerk et al., 1995) and *Rickettsia* (Andersson et al., 1998), organisms which are in the α-subdivision of the Proteobacteria and also associated with insects. In the endosymbionts of carpenter ants, the rRNA genes are also split into two transcription units (C. Sauer and R. Gross, personal communication) whereas in *Wigglesworthia* (Aksoy, 1995b) and the Enterobacteriaceae the order is 16S–23S (Berlyn, 1998). The organization of the rRNA genes into two transcription units suggests a possible relationship between *Buchnera* and the endosymbionts of carpenter ants.

The results of phylogenetic analyses involving all of the currently available *Buchnera* sequence information are presented in Fig. 2. Most of the characterized endosymbionts are from the family Aphididae. Based on 16S rDNA, *Buchnera* forms one clade within which two well-supported subclades are apparent. These are the aphids of the Aphididae and the Sc and Mr from the tribe Fordini in the family Pemphigidae. Additional studies using a portion of *trpB* (Fig. 2b) confirmed some of these relationships and provided further resolution within the genus *Uroleucon*. These relationships are in broad agreement with the results of evolutionary studies of plasmid-associated *trpE*, *leuBCD*, and *repA1* (Fig. 2c, d, e; Baumann et al., 1997b, 1999b; Bracho et al., 1995; Rouhbakhsh et al., 1996, 1997; Silva et al., 1998; van Ham et al., 1997, 1999). Within the genus *Uroleucon*, the phylogeny based on *trpB* is in good agreement with the more extensive analysis of host phylogeny based on mitochondrial and nuclear genes (Clark et al., 2000; Moran et al., 1999).

The congruence of phylogenies derived from *Buchnera* chromosomal and plasmid genes, as well as host mitochondrial and nuclear genes, is strong evidence for a vertical mode of evolution with no exchange of either bacteria or plasmids among host lineages (Moran and Baumann,

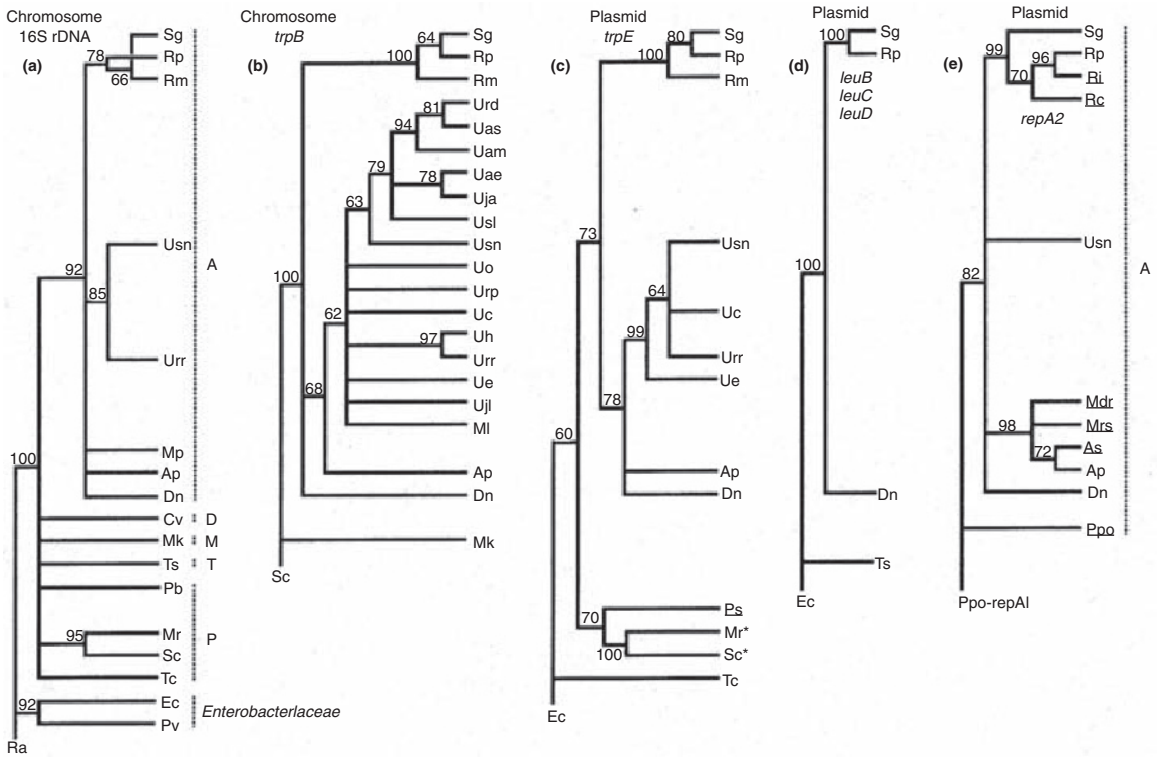

Fig. 2. Phylogenetic trees resulting from parsimony analyses using Buchnera (a) 16S rDNA, (b) trpB, (c)trpE, (d) leuB, leuC, and leuD, and (e) repA1. Numbers at nodes are bootstrap percentages from parsimony searches (1000 replicates). Abbreviations designating the insect hosts and are given in Table 2. In (a), Enterobacteriaceae: Ec(E. coli), Pv(P. vulgaris); Ra(Ruminobacter amylophilus). Dashed lines in (a) designate aphid species within one family: A, Aphididae; D, Drepanosiphidae; M, Mindaridae; T, Thelaxidae; P, Pemphigidae. Dashed line in (e) designates aphids within one family. Underlined abbreviations in (c) and (e) refer to aphid species not included in the other analyses. * in (c) designates chromosomal genes. For references see text.

Table 2. Abbreviations of aphid species used in this chapter.

Abbreviation	Aphid species	Family	Tnbe
Rc	*Rhopalosiphum cerasifoliae*	Aphididae	Aphidini
Ri	*Rhopalosiphum insertum*	Aphididae	Aphidini
Rm	*Rhopalosiphum maidis*	Aphididae	Aphidini
Rp	*Rhopalosiphum padi*	Aphididae	Aphidini
Sg	*Schizaphis graminum*	Aphididae	Aphidini
Ap	*Acyrthosiphon pisum*	Aphididae	Macrosiphini
As	*Aulacorthum solani*	Aphididae	Macrosiphini
Dn	*Diuraphis noxia*	Aphididae	Macrosiphini
Ml	*Macrosiphoniella ludovicianae*	Aphididae	Macrosiphini
Mp	*Myzus persicae*	Aphididae	Macrosiphini
Mdr	*Metopolophium dirhodum*	Aphididae	Macrosiphini
Mrs	*Macrosiphum rosae*	Aphididae	Macrosiphini
Uae	*Uroleucon aeneum*	Aphididae	Macrosiphini
Uam	*Uroleucon ambrosiae*	Aphididae	Macrosiphini
Uas	*Uroleucon astronomus*	Aphididae	Macrosiphini
Uc	*Uroleucon caligatum*	Aphididae	Macrosiphini
Ue	*Uroleucon erigeronense*	Aphididae	Macrosiphini
Uh	*Uroleucon helianthicola*	Aphididae	Macrosiphini
Uja	*Uroleucon jaceae*	Aphididae	Macrosiphini
Ujl	*Uroleucon jaceicola*	Aphididae	Macrosiphini
Uo	*Uroleucon abscurum*	Aphididae	Macrosiphini
Urd	*Uroleucon rudbeckiae*	Aphididae	Macrosiphini
Urp	*Uroleucon rapunculoidis*	Aphididae	Macrosiphini
Urr	*Uroleucon rurale*	Aphididae	Macrosiphini
Usl	*Uroleucon solidaginis*	Aphididae	Macrosiphini
Usn	*Uroleucon sonchi*	Aphididae	Macrosiphini
Ppo	*Pterocomma populeum*	Aphididae	Pterocommatinae
Cv	*Chaitophorus vininalis*	Drepanosiphidae	Chaitophorini
Mk	*Mindarus kinseyi*	Mindaridae	Mindarini
Mr	*Melaphis rhois*	Pemphigidae	Fordini
Sc	*Schlechtendalia chinensis*	Pemphigidae	Fordini
Pb	*Pemphigus betae*	Pemphigidae	Pemphigini
Ps	*Pemphigus spyrothecae*	Pemphigidae	Pemphigini
Tc	*Tetraneura caerulescens*	Pemphigidae	Eriosomatini
Ts	*Thelaxes suberi*	Thelaxidae	

1994). An implication of the congruence between the phylogenies of *Buchnera* and corresponding aphid hosts is that dates for branch points inferred from fossil aphids can be extended to ancestral *Buchnera* (Moran et al., 1993). A further implication is that modern *Buchnera* descend from an infection of a common ancestor of all modern aphids. From the aphid fossil record, we can infer that this infection by a free-living bacterium must have occurred at least 150–250 million years ago. The divergence in 16S rDNA of modern *Buchnera* is consistent with this hypothesis of an ancient infection followed by cospeciation of *Buchnera* and hosts.

Buchnera shows a rate of base substitution in its 16S rDNA that is about twice as great as that in related free-living bacteria based both on relative rate comparisons with free-living taxa and on comparisons of rates calibrated with respect to absolute time (Clark et al., 1999b; Moran, 1996). The elevated substitution rate of *Buchnera* relative to that of related free-living bacteria extends to genes encoding proteins (Brynnel et al., 1998; Clark et al., 1999b; Moran, 1996; Wernegreen and Moran, 1999). Based on calibrated rates for protein-coding genes, synonymous sites evolve about twice as fast and nonsynonymous sites about six times as fast in *Buchnera* as in *E. coli*/*Salmonella typhimurium*, based on an absolute time scale (Table 3). The rate differences are considerably greater on a scale based on generations, since *Buchnera* appears to have fewer generations per year than do natural populations of enteric bacteria (Clark et al., 1999b).

The most plausible explanation for the faster rate of substitution in *Buchnera* is that the population structure of *Buchnera*, involving strictly vertical transmission of a small inoculum between hosts, results in greater levels of genetic drift, which can increase the fixation rate of mildly deleterious mutations. Several observations support this explanation. First, the rate increase is found at all genes and is concentrated at sites, such as nonsynonymous sites, that are

Table 3. Substitution rates in *Buchnera* and enteric bacteria.[a]

Species pair	Estimated time of divergence	Synonymous rate		Nonsynonymous rate	
		Absolute[b]	Generation[c]	Absolute[b]	Generation[c]
Buchnera (Sg/Dn)	50–70MY	6.8–9.5	0.14–0.19	1.0–1.4	0.02–0.03
Buchnera (Sc/Mr)	50–70MY	5.1–7.2	0.17–0.24	1.1–1.6	0.04–0.05
E. coli/S. typhimurium	100–150MY	2.9–4.4	0.03–0.04	0.1–0.2	0.001–0.002

[a]Based on comparisons of over 5100 codons (Clark et al., 1999b).
[b]Substitutions/site/10° years.
[c]Substitutions/site/10° generations.

expected to be under selective constraint (Moran, 1996; Wernegreen and Moran, 1999). Second, polypeptide compositions are consistently biased towards amino acids that allow more adenine and thymine in the DNA sequence, indicating that mutational bias has affected protein evolution. Third, faster substitution rates in 16S rDNA are observed in other insect endosymbionts that share a similar transmission mode, suggesting that the endosymbiotic lifestyle has repeatedly produced the same changes in patterns of molecular evolution. Finally, the 16S rRNA secondary structure of *Buchnera* and other endosymbionts has lower thermal stability than that of related free-living bacteria, as expected if the DNA base substitutions are mildly deleterious (Lambert and Moran, 1998).

The 16S rDNA substitution rate of *Buchnera* is about 35 times greater than that of homologous regions of 18S rDNA of hosts, based on comparisons of pairwise divergences of corresponding aphid and *Buchnera* taxa (Moran et al., 1995). Thus, the hypothesis of a universal rate of substitution in rDNA is not even approximately true.

Taxonomy

The genus *Buchnera* contains one species, *Buchnera aphidicola*, and the type strain is the endosymbiont of the aphid *Schizaphis graminum* (Munson et al., 1991a). Currently this species name designates the lineage consisting of the P-endosymbionts of aphids. There are over 4,000 species of aphids (Blackman and Eastop, 1984; Remaudière and Remaudière, 1997) of which only 35 have been characterized by molecular methods. Consequently our conclusions are based on a very small sample of aphid species. Although 16S rDNA has been useful for showing the monophyletic origin of aphid endosymbiosis and the establishment of major aphid subgroups, it is far too conserved to be useful for defining relationships among endosymbionts of closely related aphids. Some success has been obtained by the use of other, less conserved, molecules (Fig. 2). The 16S rDNA sequence difference of *Buchnera* in Sg and Sc (the most distantly related aphids) is about the same as that between *E. coli* and *Proteus vulgaris*. Thus, subsequent studies using less conserved molecules will probably indicate that *Buchnera* should be subdivided into new species. So far, no studies have addressed the range of variation within endosymbionts of a single aphid species.

Habitat

Location and Ultrastructure During their reproductive phase, aphids contain within their body cavity a bilobed structure called a bacteriome consisting of 60–90 uninucleate, polyploid cells called bacteriocytes (Douglas and Dixon, 1987). These cells are filled with host-derived vesicles containing *Buchnera* (Fig. 3a). This organism is spherical or oval in shape, 2–4 m in diameter, with a cell wall consisting of two-unit membranes, as is characteristic of Gram-negative bacteria (Fig. 3b; Akhtar and van Emden, 1994; Griffiths and Beck, 1973; Hinde, 1971b; McLean and Houk, 1973). A thin layer corresponding to peptidoglycan has been detected (Houk et al., 1977). The presence of peptidoglycan also is indicated by penicillin-induced alterations of the cell wall as well as by chemical analysis (Griffiths and Beck, 1974; Houk et al., 1977).

Number of Endosymbionts Buchnera contains one copy of the 16S rRNA gene (Munson et al., 1991b, 1993; Unterman et al., 1989) and one copy of *groEL* (Ohtaka et al., 1992; Hassan et al., 1996). The number of copies of *Buchnera* 16S rRNA genes in the aphid Sg was studied by quantitative PCR (Baumann and Baumann, 1994) and was found to be $0.5–1.2 \times 10^7$ mg^{-1} aphid wet weight. Using quantitative hybridization of a *Buchnera groEL* probe, the number of genome copies in Ap was estimated at $1–2 \times 10^7$ mg^{-1} aphid wet weight (Humphreys and Douglas, 1997). In both of these studies the number of *Buchnera* cells was assumed to be identical to the number of genome copies. However a recent study has demonstrated that *Buchnera* (Ap) is polyploid, containing an average of about 120 genomes per cell (Komaki and Ishikawa, 1999). If the average number of genomes per

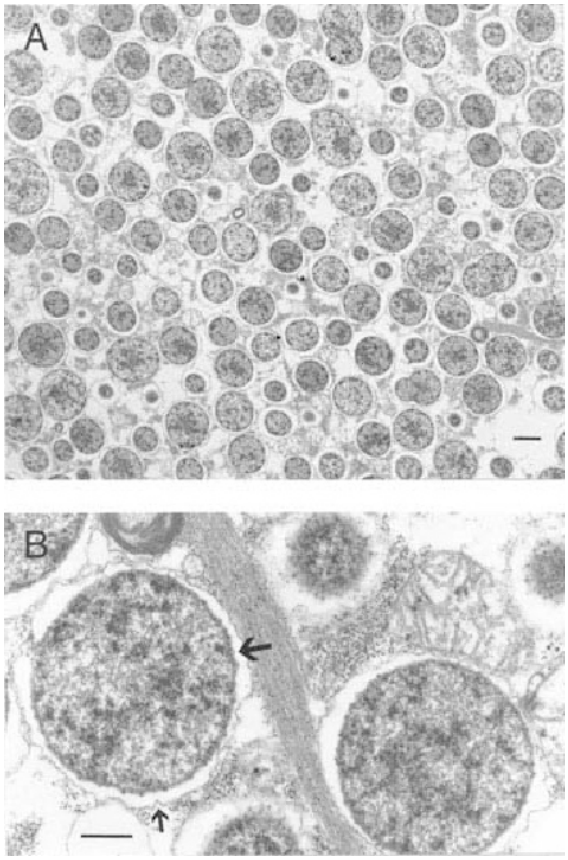

Fig. 3. Electron micrographs of *Buchnera*, the P-endosymbiont of aphids. (a) Endosymbionts within a bacteriocyte, bar = 1 m. (b) Higher magnification showing the Gram-negative cell-wall (large arrow) and the vesicle membrane (small arrow), bar = 0.5 m. Photos courtesy of Marv Kinsey and Don McLean.

endosymbiont is relatively constant, then the number of endosymbionts is about 100-fold less than estimated previously, or about 105 mg^{-1} aphid wet weight. This value is considerably lower than the estimates for Ap of $1.6–1.8 \times 10^6$ endosymbionts mg^{-1} aphid wet weight, based on microscopic enumeration of the endosymbionts (Harrison et al., 1989).

Growth and Reproduction In their most active stage, aphids are wingless females, which reproduce by parthenogenesis, giving birth to live young. There is telescoping of generations in that the mother aphid contains embryos that, in turn, may contain other embryos (Dixon, 1973, 1992). Studies on the growth of Sg (Baumann and Baumann, 1994) have indicated that newly born aphids weigh 24 μg and contain 2×10^5 copies of the *Buchnera* genome. The increase in the number of endosymbiont genomes parallels the increase in the weight of the aphid. The maximum weight is reached in 9–10 days at which time the aphid weighs 540 μg and contains 5.6×10^6 *Buchnera* genomes. The endosymbionts are partitioned between maternal and embryonic bacteriocytes. In a mature aphid, most of the *Buchnera* genomes are found in the embryos (Humphreys and Douglas, 1997). The first young are born in 8–9 days; each aphid can produce 60 to 80 live young during its lifetime. Douglas and Dixon (1987) showed that, during the period of growth, there is a concomitant increase in the maternal bacteriocyte volume as well as a small drop in bacteriocyte number. In the adult aphid the number of maternal bacteriocytes in the bacteriome undergoes a sharp decrease probably due, in part, to their dispersion within the abdomen, their degradation, as well as the degradation of *Buchnera* (Brough and Dixon, 1990; Douglas and Dixon, 1987; Griffiths and Beck, 1973; Hinde, 1971a).

Aphids may also produce sexual forms with the females depositing eggs that overwinter and hatch in the spring. *Buchnera* is maternally transmitted (transovarial transmission) to both developing embryos and eggs. Maternal bacteriocytes adjacent to an embryo near the blastoderm stage form a small opening through which the endosymbionts pass. *Buchnera* then moves through the intervening hemolymph and enters a nearby opening on the oocyte surface. During early embryonic development, the presumptive bacteriocytes form, and the endosymbionts migrate into these cells (Buchner, 1965; Blackman, 1987; Hinde, 1971a). Symbiont invasion of eggs also occurs from the dispersed bacteriocytes and they can be observed as an aggregate at the posterior pole of the mature egg (Buchner, 1965; Brough and Dixon, 1990). *Buchnera* and bacteriomes appear to be nearly universal in aphids (Buchner, 1965). However, some species of the tribe Cerataphidini lack both and instead contain yeast-like extracellular symbionts within their body cavity (Buchner, 1965; Fukatsu and Ishikawa 1992a, 1996). Some species of aphids may produce dwarf males and/or sterile female soldiers that may also lack endosymbionts (Buchner, 1965; Fukatsu and Ishikawa, 1992b; Fukatsu et al., 1994).

Physiology

Nutrition and Metabolism Plant sap, the diet of aphids, has an excess of carbohydrate relative to amino acids and other nitrogenous compounds (Dadd, 1985; Douglas, 1998; Sandström and Pettersson, 1994; Sandström and Moran, 1999). Aphids, like other insects, are thought to require 10 preformed amino acids and these essential amino acids are present in low amounts in plant sap. Some species of aphids can grow on synthetic diets even in the absence of essential amino acids. Adding antibiotics to such diets

results in the elimination of endosymbionts and the failure of the aphids to reproduce. There is some sparing effect when the essential amino acids are included in the antibiotic-containing diet. These experiments have generally been interpreted as indicating that one of the functions of *Buchnera* is the synthesis of essential amino acids for the aphid host (reviewed by Baumann et al., 1995; Douglas, 1998). A major problem is that compared with growth on plants, growth on artificial diets is poor and generally limited to a few generations. In addition, aphid growth on complete synthetic diets in the presence of antibiotics is even worse, indicating that *Buchnera* provides nutrients or functions that cannot be provided by the artificial diets. The effects of antibiotics on a number of aphid properties have been recently reviewed (Wilkinson, 1998). There is a further complication with some of the nutritional studies, in which physiological effects have been attributed to the removal of *Buchnera*. The aphid strain used may also contain S-endosymbionts, as is true of the strain of the aphid Ap used in the studies of A. E. Douglas and her collaborators (Douglas and Prosser, 1992; Wilkinson 1998). The S-endosymbiont probably does not perform any essential functions for the host (see section on S-endosymbionts of aphids in this Chapter); nevertheless, the use of antibiotics eliminates both endosymbionts, and consequently the observed effects of this loss may not be attributable solely to the loss of *Buchnera*.

Currently one of the more complete studies involves the essential amino acid tryptophan. Using a strain of Ap containing an S-endosymbiont, Douglas and Prosser (1992) have shown a sparing effect of tryptophan in chlortetracycline-containing synthetic diets on aphid growth. In addition they detected low levels of tryptophan synthase in *Buchnera* and found that activity was absent in chlortetracycline-treated and thus endosymbiont-free aphids. The assays used (Smith and Yanofsky, 1962) crude extracts of whole aphids as well as preparations enriched in endosymbionts and measured the disappearance of the substrate indole and not the appearance of the product tryptophan. Indole or indole derivatives may be substrates for a variety of reactions catalyzed by enzymes found in crude extracts of insects. No information was provided about the linearity of increasing enzyme activity with increasing crude extract concentration. In spite of possible difficulties with this assay, the dependence of the reaction on "the substrate [sic] pyridoxal phosphate" (Douglas and Prosser, 1992) is consistent with it being a measure of tryptophan synthase activity.

Using synthetic diets containing radiolabelled sulfate, it was shown that *Buchnera* can reduce this compound to the level of hydrogen sulfide and incorporate it into methionine and cysteine and that these amino acids are found in aphid tissue (Douglas, 1988). Using ^{14}C-radiolabelled amino acids, it was found that the synthesis of the essential amino acids arginine, threonine, isoleucine and lysine was reduced or eliminated by the inclusion of rifampicin in the diet (Liadouze et al., 1996). Sasaki and Ishikawa (1995) also showed that treatment of aphids with rifampicin eliminated the incorporation of dietary ^{15}N-glutamine into the essential amino acids arginine, histidine, isoleucine and/or leucine, phenylalanine, threonine, and valine.

Glutamine is the predominant amino acid in phloem and also in aphid hemolymph (Sandström and Pettersson, 1994; Sasaki et al., 1990). Isolated bacteriocytes were found to take up glutamine and convert it to glutamate, which subsequently was taken up by *Buchnera* (Sasaki and Ishikawa, 1995). Isolated endosymbionts incorporated the nitrogen of glutamine into the essential amino acids isoleucine, leucine, valine, and phenylalanine as well as a number of other amino acids, and these amino acids were excreted into the suspending medium. Whitehead and Douglas, however, could not find any evidence for excretion of amino acids by *Buchnera* (cited in Douglas, 1997).

Using synthetic diets, Nakabachi and Ishikawa (1999) demonstrated a requirement for riboflavin by rifampicin-treated aphids. These results indicate that *Buchnera* is required for the synthesis of at least one vitamin for the aphid host.

Whitehead and Douglas (1993) isolated vesicles containing *Buchnera* and showed that they readily took up acetic, glutamic and aspartic acid as well as tricarboxylic acid cycle intermediates and oxidized them to CO_2. Oxygen consumption was also detected and was greatly reduced by KCN. These results suggest the presence of a tricarboxylic acid cycle in the endosymbionts and indicate a respiratory metabolism. The latter conclusion is consistent with the presence of a gene for a subunit of NADH dehydrogenase I, an enzyme involved in the generation of a proton motive force during respiration, and of all the genes for ATP synthase, a membrane-associated enzyme which utilizes the proton motive force for the synthesis of ATP (Table 4).

Gene Expression *Buchnera* messenger RNA (mRNA) has been detected for a variety of genes encoding proteins involved in amino acid biosynthesis (Table 4). This includes genes for amino acids of the glutamate (*argA*) and aspartate (*thrB*) families (Nakabachi and Ishikawa, 1997), the shikimate pathway (*aroH*), as well as the biosynthetic pathway for tryptophan (*trpE*, *trpD*,

Table 4. Genes of *Buchnera* from the aphid *S. graminum*.[a]

Gene symbol	Gene product description	Linkage group[b]
I. SMALL-MOLECULE METABOLISM		
B. Energy metabolism		
1. Glycolysis		
gap A	Glyceraldehyde-3-phosphate dehydrogenase	13
tpiA	Triosephosphate isomerase	3
5. Pentose phosphate pathway		
a. Oxidative branch		
gnd	Gluconate-6-phosphate dehydrogenase	2
7. Respiration		
a. Aerobic		
nuoC(D)[c]	NADH dehydrogenase I, subunits cd	
c. Electron transport		
fdx	Ferredoxin	1
fpr	Ferredoxin-NADP reductase	12
9. ATP proton motive force		
atp A	ATP synthase, α-subunit	1
atp B	ATP synthase, subunit a	1
atp C	ATP synthase, ε-subunit	1
atp D	ATP synthase, β-subunit	1
atp E	ATP synthase, subunit c	1
atp F	ATP synthase, subunit b	1
atp G	ATP synthase, γ-subunit	1
atp H	ATP synthase, -subunit	1
D. Amino acid biosynthesis		
1. Glutamate family		
argA[d]	N-acetylglutamate synthase	
argH	Argininosuccinate lyase	7
2. Aspartate family		
dapD	Succinyl-diaminopimelate aminotransferase	4
thrA	Aspartokinase I	
thrB[d]	Homoserine kinase	
3. Serine family		
cysE	Serine acetyltransferase	7
serC	Phosphoserine amino transferase	3
4. Aromatic amino acid family		
aroA	5-Enolpyruvylshikimate-3-phosphate synthase	3
aroC	Chorismate synthase	2
aroE	Dehydroshikimate reductase	8
aroH	3-deoxy-*D-arabino*-heptulosonate-7-phosphate synthetase (DAHP synthetase)	10
trpA	Tryptophan synthase, A protein	5
trpB	Tryptophan synthase, B protein	5
trpC(F)	Indole-3-glycerolphosphate synthetase and *N*-(5-phosphoribosyl) anthranilate isomerase	5
trpD	Phosphoribosylanthranilate transferase	5
trpE (p)[e]	Anthranilate synthase, A subunit	16
trpG (p)[e]	Anthranilate synthase, B subunit (glutamine amidotransferase)	16
5. Histidine		
hisA	*N*-(5′-phospho-L-ribosyl-formimino)-5-amino-1-(5′-phosphoribosyl)-4-imidazolecarboxamide isomerase	2
hisB	Imidazoleglycerolphosphate dehydratase and histidinol phosphate phosphatase	2
hisC	Histidinol-phosphate aminotransferase	2
hisD	Histidinol dehydrogenase	2
hisF	Imidazoleglycerol phosphate synthase subunit (with HisH)	2
hisG	ATP phosphoribosyl transferase	2
hisH	Glutamine amidotransferase subunit (with HisF)	2
hisI	Phosphoribosyl-AMP cyclohydrolase and phosphoribosyl-ATP pyrophosphatase	2
7. Branched-chain family		
ilvC	Acetohydroxy acid isomeroreductase	1
ilvD	Dihydroxyacid dehydratase	1
ilvH	Acetohydroxyacid synthase, small subunit	4
ilvI	Acetohydroxyacid synthase, large subunit	4
leuA (p)[e]	2-Isopropylmalate synthase	15
leuB (p)[e]	3-Isopropylmalate dehydrogenase	15
leuC (p)[e]	Isopropylmalate isomerase subunit	15
leuD (p)[e]	Isopropylmalate isomerase subunit	15

(Continued)

Table 4. *Continued*

Gene symbol	Gene product description	Linkage group[b]
F. Purines, pyrimidines, nucleosides, and nucleotides		
	3.2′-Deoxyribonucleotide metabolism	
dcd	2′-Deoxycytidine 5′-triosephosphate deaminase	2
trxB	Thioredoxin reductase	3
G. Biosynthesis of cofactors, prosthetic groups, and carriers		
	9. Riboflavin	
ribE[d]	Riboflavin synthase, β-chain	
	10. Thioredoxin, glutaredoxin, and glutathione	
trxA	Thioredoxin	1
II. BROAD REGULATORY FUNCTIONS		
rpoD	RNA polymerase, σ^{70} subunit	7
rpoH[d]	RNA polymerase, σ^{32} subunit, regulation of proteins induced at high temperature	
III. MACROMOLECULE METABOLISM		
A. Synthesis and modification of macromolecules		
	1. rRNA and "stable" RNAs	
rrf	5S rRNA	8
rrl	23S rRNA	8
rrs	16S rRNA	11
	2. Ribosomal protein synthesis and modification	
rplL	50S ribosomal protein L7/L 12	9
rplT	50S ribosomal protein L20	10
rpmH	50S ribosomal protein L34	1
rpmI	50S ribosomal protein L35	10
rpsA	30S ribosomal protein S1	3
rpsD	30S ribosomal protein S4	14
rpsK	30S ribosomal protein S11	14
	4. tRNAs	
tRNA^{Glu}	Glutamate-tRNA	8
tRNA^{Phe}	Phenylalanine-tRNA	1
tRNA^{Trp}	Tryptophan-tRNA	1
	5. Aminoacyl tRNA synthetases and their modification	
argS	Arginine tRNA synthetase	11
aspS	Aspartic tRNA synthetase	3
cysS	Cysteine tRNA synthetase	8
metG	Methionine tRNA synthetase	2
serS	Serine tRNA synthetase	3
thrS	Threonine tRNA synthetase	10
trmE	tRNA methyltransferase	1
	7. DNA replication, restriction/modification, and recombination	
dnaA	DNA biosynthesis, initiation of chromosome replication, global transcription regulator	1
dnaG	DNA biosynthesis, DNA primase	7
dnaN	DNA polymerase III holoenzyme, β-subunit	1
dnaQ	DNA polymerase III holoenzyme, ε-subunit	11
gidA	Chromosome replication?	1
gyrB	DNA gyrase subunit B	1
himD	Integration host factor, β-subunit	3
rep	Rep helicase, ssDNA-dependent ATPase	1
	8. Protein translation and modification	
efp[f]	Elongation factor EF-P	
infC	Initiation factor IF-3	10
tuf[g]	Elongation factor EF-Tu	
	9. RNA synthesis, RNA modification, and DNA transcription	
rho	Transcription termination factor Rho	1
rpoA	RNA polymerase, α-subunit	14
rpoB	RNA polymerase, β-subunit	9
rpoC	RNA polymerase, β′-subunit	9
	11. Phospholipids	
clh[h]	Cardiolipin synthase	
B. Degradation of macromolecules		
	1. RNA	
rnh	RNase H	11
rnpA	RNase P	1
	3. Proteins, peptides, and glycopeptides	
hslU	Heat shock protein, protease?	12
htrA	Periplasmic serine protease and heat shock protein	4
soh[i]	Periplasmic protease	

Table 4. *Continued*

Gene symbol	Gene product description	Linkage group[b]
C. Cell envelope		
2. Surface polysaccharides, lipopolysaccharides, and antigens		
kdtB	Putative enzyme of lipopolysaccharide synthesis	12
4. Murein sacculus and peptidoglycan		
ddlB	D-alanine-D-alanine ligase	6
murC	L-alanine-adding enzyme, UDP-*N*-acetyl-muramate-alanine ligase	6
murE	*meso*-Diaminopimelate-adding enzyme	4
IV. CELL PROCESSES		
B. Chaperones		
dnaJ[d]	Heat shock protein	
dnaK[d]	Heat shock protein (Hsp 70), DNA biosynthesis	
groEL	Heat shock protein (Hsp 60)	1
groES	Heat shock protein (Hsp 10)	1
hscA	Cold shock protein (Hsp 70)	1
hscB	Cold shock protein	1
C. Cell division		
ftsA	Cell division protein, complexes with FtsZ	6
ftsI	Septum formation; penicillin binding protein 3; peptidoglycan synthase	4
ftsL	Cell division protein; ingrowth of wall at septum	4
ftsZ	Cell division, forms circumferential ring	6
E. Protein and peptide secretion		
secB	Protein export, molecular chaperone	7
V. OTHER		
F. Adaptations and atypical conditions		
ibp	Heat shock protein, HSP20 family	12
ADDITIONAL ORFs		
bcp	*E. coli homolog* Bacterioferritin comigratory protein 1788825	2
nifS	1788879	1
ybN	1790040	7
yjEA	Hypothetical lysine tRNA synthase homolog 1790599	12
10kDA	YIDD_ECOLI	1
39kDa	1790589	1
60kDa	1790140	1
ORF113	1786351	6
ORF128	1788878	1
ORF177	1788671	2
ORF194	1788860	1
ORF217	1787362	13
ORF235	1786354	6
ORF312	1786270	4
ORF340	1788543	4
ORF453	1788858	1
ORFI	1786406	11
ORFV	1787508	5
ORFVI	1787507	5
P14	1787506	5
ORFA	1788269 Transmembrane protein?	5
ORFB	1787524	5
ORFC	1787361 ABC transporter protein? ATP-binding site?	13
ORFD	1789158	13
pLeu		
repA1 (p)[e]	Related to RepA protein of IncFII plasmids	15
repA2 (p)[e]	Related to RepA protein of IncFII plasmids	15
ORF1 (p)[e]	Related to *E. coli* 1789376	15

[a]Gene list arranged according to the classification of gene products of Riley and Labedan (1996). Genes from *Buchnera* from other species of aphids are indicated.
[b]See Table 5.
[c]From *Buchnera* (Usn).
[d]From *Buchnera* (Ap) (Nakabachi and Ishikawa, 1997, 1999; Sato and Ishikawa, 1997a, b).
[e]p, plasmid associated gene.
[f]From *Buchnera* (Mp) (Hogenhout et al., 1998).
[g]From Brynnel et al. (1998).
[h]From *Buchnera* (Dn, Usn).
[i]From *Buchnera* (Sc) (Lai et al., 1995).

trpA), branched-chain amino acids (*ilvI*, *ilvD*, *leuA*), and histidine (*hisG*; Baumann et al., 1999a). *Buchnera* mRNA has been detected also for a gene involved in the biosynthesis of riboflavin (*ribE*; Nakabachi and Ishikawa, 1999) as well as for genes involved in the heat shock response (*groEL*, *groES*, *dnaK*, *dnaJ*; Table 4; Sato and Ishikawa, 1997a, b). Numerous *Buchnera* proteins have also been detected by immunological methods. These include *GroEL*, *GroES*, and *DnaK* (Kakeda and Ishikawa, 1991; Sato and Ishikawa, 1997b) as well as ribosomal protein S1 (the product of *rpsA*; Clark et al., 1996) and the protein involved in septum formation during cell division (the product of *ftsZ*; Table 4; Baumann and Baumann, 1998).

In bacteria, rRNA genes are transcribed from strong promoters. Comparisons of the regions upstream of rRNA genes from six species of *Buchnera* indicated conservation of sequences resembling the –35 and –10 regions of σ^{70} promoters as well as boxA and boxC (Munson et al., 1993; Rouhbakhsh, 1995). Similar putative –35 and –10 regions were found in *Buchnera* plasmids containing genes for tryptophan and leucine biosynthesis (Baumann et al., 1999b; Rouhbakhsh et al., 1996; Silva et al., 1998).

GroEL Overproduction and Its Significance In *Buchnera* the chaperonin, GroEL, constitutes a major fraction of the total protein (Sato and Ishikawa, 1997a). In addition, GroEL is present in aphid hemolymph (van den Heuvel et al., 1994). Overproduction of GroEL is a characteristic of some endosymbionts and pathogens in the intracellular environment (Hogenhout et al., 1998). This protein mediates the folding of peptides into their functional forms as well as the repair of damaged proteins (Gross, 1996). *Buchnera* GroEL is able to complement *E. coli* mutants (Ohtaka et al., 1992). GroEL has been localized in maternal and embryonic *Buchnera* by immunohistochemistry (Fukatsu and Ishikawa, 1992c). Electron micrographs indicate that the purified *Buchnera* GroEL has the characteristic double-ring appearance observed with the *E. coli* protein (Filichkin et al., 1997; Hara and Ishikawa, 1990). The endosymbiont protein has ATPase activity and in the presence of *E. coli*, GroES could reconstitute denatured *Rhodospirillum rubrum* ribulose-1,5-biphosphate carboxylase (Kakeda and Ishikawa, 1991).

The *Buchnera groESL* operon organization resembles that of *E. coli* (Hassan et al., 1996; Hogenhout et al., 1998; Ohtaka et al., 1992). Upstream of *groES* are nucleotide sequences characteristic of the –35 and –10 regions of σ^{32} promoters. A message of 2.1 kb (containing both *groES* and *groEL*) is made by the endosymbiont using only this promoter (Sato and Ishikawa, 1997a). It is not understood why GroES is low in the endosymbiont, in contrast to the high quantities of GroEL (Kakeda and Ishikawa, 1991). The genes for σ^{32} (*rpoH*) as well as *dnaKJ* have been cloned and sequenced (Sato and Ishikawa, 1997a, b). The latter also are transcribed solely from a σ^{32} promoter. In *E. coli* as well as other organisms, transcription of the *groESL* operon and the *dnaKJ* operon is part of the σ^{32} regulon, and their synthesis is increased by heat shock (Gross, 1996). It would appear that this mode of regulation is modified in *Buchnera* (Sato and Ishikawa, 1997a, b). Synthesis of *groESL* and *dnaKJ* mRNA is constitutive and is not increased by heat shock. This conclusion is supported by the observation that there is no increase in the level of total GroEL in aphids shifted from 23 to 33°C for one day (Baumann et al., 1996).

Baumann et al. (1996) arrived at an estimate of the amount of GroEL per *Buchnera* cell, based on the premise that the endosymbiont contained only one genome copy. Recently it has been shown that *Buchnera* is polyploid, containing an average of 120 genome copies per cell (Komaki and Ishikawa, 1999). If this result is incorporated into the calculation, an impossible excess of GroEL would be present in each endosymbiont cell. Since protein extracts of whole aphids were used in the estimation of GroEL content, this result could be explained by the finding that GroEL is also present in the hemolymph (van den Heuvel et al., 1994).

Perhaps the major economic effect of aphids on agriculture is their ability to transmit plant viruses (Blackman and Eastop, 1984; Gray and Banerjee, 1999; Sylvester, 1985). *Buchnera*-derived GroEL has been implicated in the survival of luteoviruses in the hemolymph (Filichkin et al., 1997; van den Heuvel et al., 1994; Hogenhout et al., 1998). These viruses replicate in the plant and are ingested by aphids when they feed on phloem sap. Subsequently they are transported from the digestive tract into the hemolymph and from there, into the salivary gland for transmission to plants via salivary secretions. The viruses are retained in an infective form (without replication) in the hemolymph throughout the life span of the aphid. There is evidence that the GroEL that is found in the hemolymph coats the virus particles and protects them from host defenses. A region in *Buchnera* GroEL has been identified which is essential for binding to the virus (Hogenhout et al., 1998), and similarly a portion of a viral capsid protein has been identified as the region to which the endosymbiont GroEL binds (van den Heuvel et al., 1997). Transmission of plant viruses may be advantageous to the aphid because infected plants have higher levels of nutrients in their sap (Blua et al., 1994).

An additional reason for the constitutive synthesis of high amounts of GroEL by *Buchnera* may be to compensate for the accumulated amino acid substitutions which have occurred at a high rate in this endosymbiont (Moran, 1996; Table 3). These slightly deleterious changes may result in proteins of decreased stability, and the high levels of GroEL may compensate for these changes, allowing proper folding and retention of function. A similar role for chaperones in masking deleterious mutations has been recently suggested on the basis of work on *Drosophila* heat shock protein (Pennisi, 1998; Rutherford and Lindquist, 1998).

Genetics

Genome Analysis The guanine + cytosine (G+C) content of *Buchnera* is about 28 mol% (Clark et al., 1998a; Ishikawa, 1987). The genome size of the endosymbiont from the aphid Ap has been found to be 0.657 Mb (Charles and Ishikawa, 1999). This is considerably below such free-living organisms as *E. coli* (4.6 Mb; Blattner et al., 1997) and *Haemophilus influenzae* (1.8 Mb; Fleischmann et al., 1995) and the intracellular pathogens, *Chlamydia trachomatis* (1.0 Mb; Stephens et al., 1998) and *Rickettsia prowazekii* (1.1 Mb; Andersson et al., 1998). The *Buchnera* genome is somewhat larger than that of the pathogen *Mycoplasma genitalium* (0.58 Mb; Fraser et al., 1995). An unusual property of the *Buchnera* genome is that it appears to be present as about 120 copies per cell (Komaki and Ishikawa, 1999).

Approximately 130 kb of DNA have been sequenced from *Buchnera* (from Sg) (Baumann and Baumann, 1998; Baumann et al., 1995; Clark et al., 1998a, b; Thao and Baumann, 1998). The choice of this aphid was predicated on the fact that it contains only one endosymbiont as indicated by morphological examinations as well as extensive restriction enzyme and Southern blot analysis of whole aphid DNA, using probes for many different genes. The latter results indicated that, with the exception of plasmid amplified DNA, only one copy of the targeted genes was present in the endosymbiont genome. In many cases, the DNA that was used for cloning was also endosymbiont-enriched. There were many independently cloned DNA fragments with identical overlapping sequences, indicating that the aphids did not contain several closely related endosymbionts. At least 20 kb of DNA also have been sequenced from *Buchnera* of each of the aphids Dn, Sc and Mr (Baumann et al., 1998a; Clark et al., 1999b; Lai et al., 1995, 1996). Table 4 lists, under different functional categories, the genes found in *Buchnera*, primarily in (Sg). The order of these genes in the DNA fragments is presented in Table 5. A total of 126 open reading frames were detected, of which 101 corresponded to *E. coli* genes with known function. The remaining 25 open reading frames all had homologs of no known function in the *E. coli* chromosome (Blattner et al., 1997). Table 6 presents the codon usage of the *Buchnera* structural genes. As expected from the G+C content, there is a strong bias for A and T, especially in the third codon position. This bias also affects the composition of proteins, favoring amino acids for which

Table 5. Order of genes on DNA fragments of *Buchnera* from the aphid *S. graminum*.[a]

Chromosomal genes

1) (34.7kb, AF008210) *39 kDa-groEL-groES-tRNA^{Phe}-trmE-60 kDa-rnpA-rpmH-dnaA-dnaN-gyrB-atpCDGAHFEB-gidA-ORF194-0RF453-fdx-hscA-hscB-ORF128-nifS-tRNA^{Trp}-ilvD-ilvC-rep-trxA-rho*
2) (12.8 kb, AF067228) *bcp-aroC-ORF177-hisG-hisD-hisC-hisB-hisH-hisA-hisF-hisI-gnd-dcd-metG*
3) (11.5 kb, L43549) *aspS-trxB-serS-serC-aroA-rpsA-himD-tpiA*
4) (9.7 kb, AF060492) *dapD-htrA-ORF340-IlvI-ilvH-ORF312-ftsL-ftsI-murE*
5) (8.4 kb, Z19055) *ORFB-ORFA-trpD-trpC(F)-trpB-trpA-ORFV-ORFVI-P14*
6) (6.8 kb, AF012886) *murC-ddlB-ftsA-ftsZ-ORF113-ORF235*
7) (6.5 kb, M90644) *dnaG-rpoD-cysE-secB-yibN-argH*
8) (6.1 kb, U09230) *aroE-tRNA^{glu}-rrl-rrf-cysS*
9) (5.0 kb, Z11913) *rplL-rpoB-rpoC*
10) (4.5 kb, U11066) *aroH-thrS-infC-rpmI-rplT*
11) (4.4 kb, L18927) *argS-rrs-ORF1-rnh-dnaQ*
12) (4.1 kb, AF108665) *hslU-ibp-fpr-yjeA-kdtB*
13) (3.9 kb, U11045) *ORFC-ORF217-gapA-ORFD*
14) (0.9 kb, M74510) *rpsK-rpsD-rpoA*

Plasmid-associated genes

15) (8.0 kb, AF041836) *leuA-leuB-leuC-leuD-repA1-ORF1-repA2*
16) (3.6 kb, Z21938) *trpEG*

[a]Numbers followed by parenthesis indicate linkage groups, numbers within parentheses indicate size of fragment and GenBank number. See Table 4 for description of gene products.

Table 6. Codon usage of *Buchnera* from the aphid *S. graminum*.[a]

AA	Codon	Fraction	AA	Codon	Fraction
Phe	UUU -	0.933	Ala	GCA -	0.470
Phe	UUC -	0.067	Ala	GCG -	0.052
Leu	UUA -	0.663	Tyr	UAU -	0.854
Leu	UUG -	0.091	Tyr	UAC -	0.146
Leu	CUU -	0.132	His	CAU -	0.865
Leu	CUC -	0.011	His	CAC -	0.135
Leu	CUA -	0.087	Gln	CAA -	0.887
Leu	CUG -	0.016	Gln	CAG -	0.113
Ile	AUU -	0.576	Asn	AAU -	0.863
Ile	AUC -	0.075	Asn	AAC -	0.137
Ile	AUA -	0.349	Lys	AAA -	0.918
Met	AUG -	1.000	Lys	AAG -	0.082
Val	GUU -	0.474	Asp	GAU -	0.875
Val	GUC -	0.054	Asp	GAC -	0.125
Val	GUA -	0.407	Glu	GAA -	0.913
Val	GUG -	0.065	Glu	GAG -	0.087
Ser	UCU -	0.448	Cys	UGU -	0.826
Ser	UCC -	0.039	Cys	UGC -	0.174
Ser	UCA -	0.273	Trp	UGG -	1.000
Ser	UCG -	0.034	Arg	CGU -	0.348
Ser	AGU -	0.177	Arg	CGC -	0.045
Ser	AGC -	0.029	Arg	CGA -	0.148
Pro	CCU -	0.456	Arg	CGG -	0.013
Pro	CCC -	0.069	Arg	AGA -	0.416
Pro	CCA -	0.420	Arg	AGG -	0.030
Pro	CCG -	0.055	Gly	GGU -	0.464
Thr	ACU -	0.474	Gly	GGC -	0.042
Thr	ACC -	0.041	Gly	GGA -	0.446
Thr	ACA -	0.433	Gly	GGG -	0.048
Thr	ACG -	0.052	Ter	UAA -	0.887
Ala	GCU -	0.426	Ter	UAG -	0.094
Ala	GCC -	0.052	Ter	UGA -	0.019

[a]Based on 19037 codons (Clark et al., 1998a).

codons contain more A and T (Moran, 1996; Clark et al., 1999b).

Buchnera was found to contain *dnaA*, encoding a protein which initiates bidirectional chromosome replication, and *ftsZ*, encoding a protein involved in septum formation during cell division (Baumann and Baumann, 1998; Lai et al., 1992a). Among other genes that were found are those encoding proteins for: peptidoglycan synthesis, cell division, DNA replication, DNA transcription, ribosomal proteins, amino acid tRNA synthases, ATP synthase, electron transport, protein secretion and glycolysis. In addition, genes for three tRNAs were detected. Genes encoding homologs of proteins involved in the *E. coli* heat shock response (*groEL*, *groES*, *htrA*, *dnaK*, *dnaJ*) and the cold shock response (*hscA*, *hscB*) were also detected (Clark et al., 1998a; Hassan et al., 1996; Ohtaka et al., 1992; Sato and Ishikawa, 1997b). Nakabachi and Ishikawa (1999) detected a gene (*ribE*) encoding a protein involved in riboflavin biosynthesis. In addition some of the genes encoding enzymes for the biosynthesis of aromatic amino acids (shikimate pathway, tryptophan branch), branched-chain amino acids (isoleucine, valine, leucine), lysine, cysteine and serine as well as genes for the complete pathway of histidine biosynthesis were found (Clark et al., 1998a, b; Thao and Baumann, 1998). The presence of genes for enzymes of amino acid biosynthesis is in marked contrast to the obligate intracellular pathogens *Rickettsia prowazekii* and *Chlamydia trachomatis*, as well as such fastidious organisms as *Mycoplasma genitalium* and *Borrelia burgdorferi*, all of which lack genes encoding enzymes of amino acid biosynthesis (Andersson et al., 1998; Fraser et al., 1995, 1997; Stephens et al., 1998). Retention of amino acid biosynthetic genes by *Buchnera* probably reflects the role of these pathways in the mutualistic association with the host aphids. Overall, these results on gene content indicate that *Buchnera* has many of the properties of free-living bacteria and would appear to be, in many respects, a self-contained, physiologically autonomous unit enclosed within bacteriocyte-derived vesicles.

Currently the most interesting comparison of the *Buchnera* genome is with the recently sequenced obligate intracellular pathogen *R. prowazekii*, an organism which is a member of the α-subdivision of the Proteobacteria (Andersson et al., 1998). Compared to the sequenced bacterial genomes, this organism is unusual in that 24% of its DNA is noncoding. In addition it has a number of pseudogenes. These findings are interpreted as a stage in the adaptation of *R. prowazekii* to an intracellular lifestyle, involving the loss of genes encoding metabolic pathways for products that are provided by the host. Currently about 20% of the *Buchnera* genome has been sequenced, and the organization of the genes in these genome fragments is highly compact and similar to that of other bacteria (Clark et al., 1998a). These findings indicate that if, as seems probable, *Buchnera* originated from an organism with a larger genome (Charles and Ishikawa, 1999), then the reduction in the genome size has already been accomplished and what has been retained is the essential gene complement required for the endosymbiotic association.

Plasmid-Associated Amplification of Biosynthetic Genes Some species of aphids have *Buchnera* in which genes for enzymes of amino acid biosynthesis are amplified on plasmids (Bracho et al., 1995; Lai et al., 1994; van Ham et al., 1999). This plasmid-associated gene amplification has been interpreted as an adaptation of *Buchnera* to an endosymbiotic association in which one of its functions is the overproduction of amino acids. This interpretation is based on analogies with other prokaryotic systems in which gene amplification is viewed as an attribute of prokaryotic

genome plasticity allowing the organism to adapt to new environments (for recent reviews see Romero and Palacios, 1997; Roth et al., 1996). Currently *Buchnera* has been found to contain three different types of plasmids. The properties of these plasmids are summarized in Table 7. The plasmid-amplified genes encode the first enzyme of the tryptophan biosynthetic pathway (TrpEG) and four enzymes (LeuA, LeuB, LeuC, LeuD) of the leucine portion of the branched-chain amino acid biosynthetic pathway. For purposes of grouping of plasmid types and ease of presentation, we have used the plasmid designations given in Table 7 followed by the abbreviation corresponding to the aphid species (Table 2).

Table 7. Designations and characteristics of *Buchnera* plasmids.[a]

1) **pTrpEG.** Two or more DnaA boxes in a putative origin of replication (Fig. 5a–h). Arrangement of DnaA boxes varies, one conserved pattern is recognized and designated at *ori*-3.6. (Fig. 5a–c). The plasmids usually consist of tandem repeats of identical or similar units containing gene(s) for a putative anthranilate synthase (TrpEG), the first enzyme of the tryptophan biosynthetic pathway.
2) **pTrpEG-R.** Plasmid contains genes for putative replication initiation proteins (*repAC1, repAC2*) which are related to replication initiation proteins of broad host range plasmids of the IncA/C group (Fig. 5i, 7c). Within the DNA encoding the C-terminal end of the replication initiation proteins and/or downstream of this gene are 19 nt-long repeats which correspond to putative interons (Fig. 5i). Plasmid consists of tandem repeats of similar units and contains genes for a putative anthranilate synthase (TrpEG), the first enzyme of the tryptophan biosynthetic pathway.
3) **pLeu.** Plasmid contains genes for putative replication initiation proteins (*repA1, repA2*) which are related to replication initiation proteins of plasmids of the IncFII group (Fig. 9a-c). Putative origin of replication downstream of *repA1*. Plasmids contain one copy of genes encoding for enzymes of leucine biosynthesis (*leuA, leuB, leuC, leuD*).

[a]For references see text.

When first used, the designation is followed by the original plasmid name given in parentheses (if applicable). There are a number of other examples of plasmid amplification of biosynthetic genes. Amplification has been interpreted as a means of increasing the rate of synthesis of endproducts. These include genes of purine biosynthesis in *Borrelia* (Margolis et al., 1994), cysteine biosynthesis in *Synechococcus* (Nicholson et al., 1995), and histamine biosynthesis in *Vibrio* (Barancin et al., 1998).

The trpEG-Containing Plasmids and Gene Silencing Figure 4 is an outline of the aromatic amino acid biosynthetic pathway. It consists of a common portion leading to chorismate (shikimate pathway) and branches to 1) phenylalanine and tyrosine as well as 2) tryptophan. In the shikimate pathway, arrows that have designations correspond to genes detected in *Buchnera* (Sg). The activity of the tryptophan biosynthetic pathway is regulated by anthranilate synthase (TrpEG) which is feedback inhibited by tryptophan (Crawford, 1989). In *Buchnera* from 11 species of aphids, *trpEG* has been found to be plasmid-associated (Fig. 5; Baumann et al., 1997b; Lai et al., 1994, 1996; Rouhbakhsh et al., 1996, 1997; van Ham et al., 1999). The remaining genes of the pathway [*trpDC(F)BA*] have been found to have a chromosomal location in all cases examined [*Buchnera* (Sg, Dn, Sc, Mr)] (Baumann et al., 1998a; Clark et al., 1999b; Lai et al., 1995). In contrast to the situation in Aphididae, in *Buchnera* (Sc, Mr), *trpEG* is not plasmid-associated but is present as one copy on the endosymbiont chromosome (Fig. 4; Clark et al., 1999b; Lai et al., 1995).

The structure of plasmids of the pTrpEG-type usually consists of tandem repeats of a nearly identical unit (Fig. 5a–h). In *Buchnera* (Sg, Rp) the plasmids contain four tandem repeats of a 3.6 kb unit, in *Buchnera* (Rm) the plasmid consists of one 3.6 kb unit, while in *Buchnera* (Ap) plasmids containing 5, 6, or 10 tandem repeats

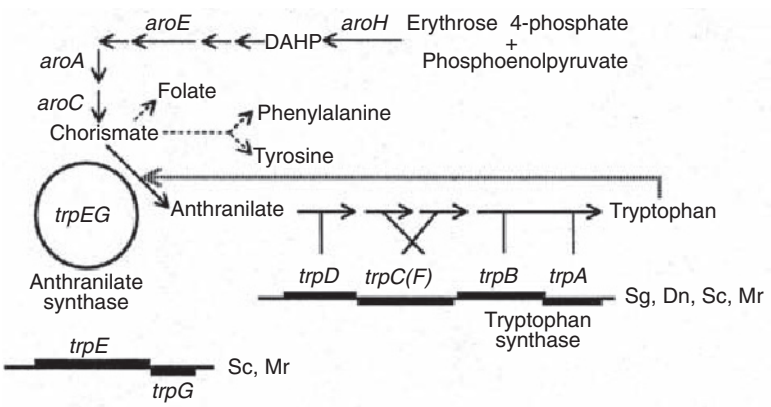

Fig. 4. Outline of the pathway for the biosynthesis of aromatic amino acids. Arrows, single enzymatic reactions; dashed arrows, several enzymatic reactions; striped arrow, feedback inhibition; genetic designations above striped line, genes detected in *Buchnera* (Sg); other genes, detected in *Buchnera* from the designated host aphids; circle, plasmid containing one or multiple copies of trpEG; DAHP, 3-deoxy-D-arabinoheptulosonate 7-phosphate. For a description of genes see Table 4; for references see text.

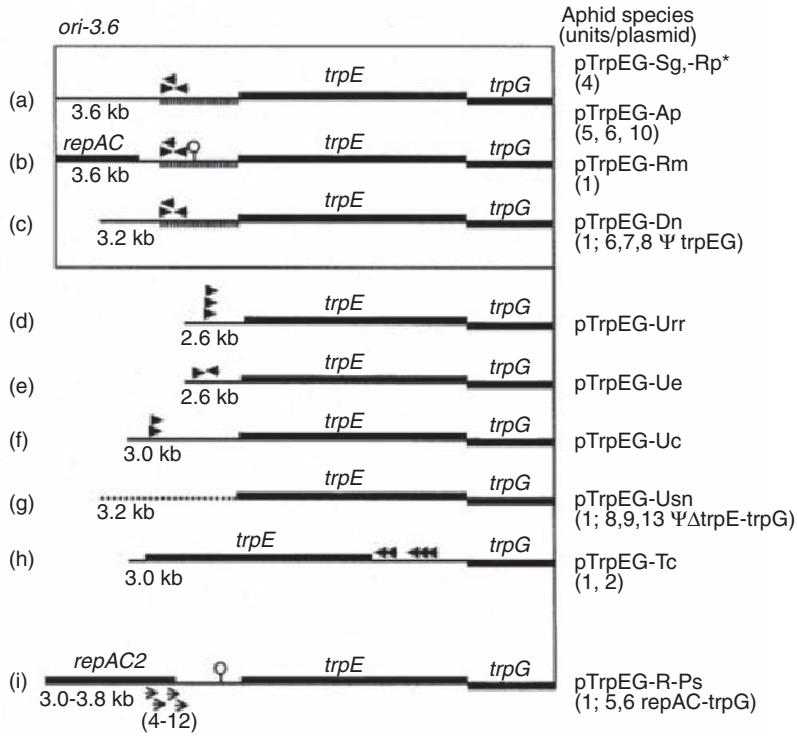

Fig. 5. Genetic maps of the repeated units which constitute trpEG-containing plasmids. Filled arrowheads, position and direction of DnaA boxes which are components of a putative origin of replication; circle on stem in (b) and (i), position of a 19 nt sequence similar to the interon of the broad host range plasmid RA1; ori-3.6, putative origin of replication found primarily in plasmids consisting of 3.6 kb repeat units (boxed); striped line, conserved sequence; dashed line, DNA that has not been sequenced; arrow in (i), 19 nt repeated sequence corresponding to a putative interon; Rp*, TrpEG-Rp also contains a remnant of repAC. For references see text.

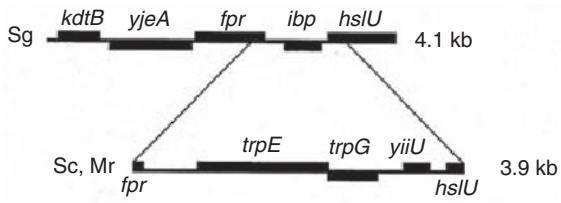

Fig. 6. Genetic map of similar chromosomal DNA fragments from Buchnera (Sg) as well as (Sc, Mr). Buchnera (Sg) lacks trpEG due to its presence on a plasmid while in Buchnera (Sc, Mr) trpEG is chromosomal. For a description of genes see Table 4; for references see text.

are found. *Buchnera* (Sg) contains about 4 plasmids per endosymbiont chromosome resulting in a 16-fold *trpEG* amplification. In *Buchnera* (Sc, Mr), in which *trpEG* is chromosomal, these two genes are found between *fpr* and *hslU* (Fig. 6). In *Buchnera* (Sg), in which *trpEG* is plasmid-associated, *trpEG* is absent from this chromosomal location, consistent with its transfer to the plasmid (Fig. 6). Instead *ibp* is present at this position, suggesting a concomitant or a subsequent acquisition of this gene (Clark et al., 1999a).

The plasmids in Fig. 5a–h all share in common the presence of 2–5 DnaA boxes which are 9 nt-long sequences to which the DnaA protein binds, thereby initiating chromosomal replication (Messer and Weigl, 1996). There is also considerable variation in the length of the repeated units (2.6 to 3.6 kb). Within this plasmid group, a readily recognized subset contains a unique arrangement of three DnaA boxes and a conserved region upstream of *trpEG* which has been designated as *ori-3.6* (Fig. 5a–c; Lai et al., 1996; Rouhbakhsh et al., 1996). *Buchnera* in aphids of the genus *Uroleucon* (which is derived from *Buchnera* within the cluster that has *ori-3.6*-containing plasmids, Fig. 2b, c) have *trpEG* units which show a considerable size range and substantial differences in the arrangement of the DnaA boxes (Fig. 5d–g; Baumann et al., 1997b; Rouhbakhsh et al., 1997). All of these *Buchnera* are from aphid species of the family Aphididae (Fig. 2a). Plasmid pTrpEG-Tc (pBTc2; Fig. 5h) is from an aphid within the family Pemphigidae (van Ham et al., 1999). The predominant form of this plasmid consists of one 3.0 kb unit; a minor form consists of two units. A distinctive feature is the presence of DnaA boxes between *trpE* and *trpG* (Fig. 5h) and not upstream of *trpEG* as is the case of the other plasmids (Fig. 5a–g). However, since there is considerable rearrangement of the DnaA boxes within plasmids in *Buchnera* of *Uroleucon* (Fig. 5d–g), it is plausible that the arrangement of DnaA boxes within pTrpEG-Tc is not a fundamental difference but a variation on the arrangement observed in the other *trpEG*-containing plasmids (Fig. 5a–g).

A totally different *trpEG*-containing plasmid is pTrpEG-R-Ps (pBPs2; Fig. 5i), which does not contain DnaA boxes but instead has putative

replication initiation proteins (RepAC) which are related to those of plasmids of the broad host-range group IncA/C (van Ham et al., 1999). Within the DNA encoding the C-terminal portion of *repAC* and/or downstream of it are 4–12 repeats of a 19 nt-long sequence corresponding to a putative interon. In addition, there is a single copy of a 19 nt-long sequence similar to the interon sequence of IncAC plasmid RA1 (Llanes et al., 1996). Curiously pTrpEG-Rm (Fig. 5b) contains in its DNA a gene for a putative RepAC protein and the 19 nt-long sequence similar to that found in plasmid RA1, while pTrpEG-Rp from a closely related aphid has a remnant of *repAC* (van Ham et al., 1999).

In several pTrpEG plasmids, the expression of most of the *trpEG* copies appears to be silenced (Baumann et al., 1997b; Lai et al., 1996; van Ham et al., 1999). In *Buchnera* (Dn) there are about two copies of pTrpEG-Dn for each endosymbiont genome (Lai et al., 1996). Plasmid pTrpEG-Dn consists of a single 3.2 kb unit containing an open reading frame corresponding to the putative protein TrpEG (Fig. 7a). This is followed by a 2.6 kb unit containing *trpEG* pseudogenes and 5–7 repeats of a 3.2 kb unit also containing *trpEG* pseudogenes. (By pseudogenes, we mean segments of DNA which are clearly recognizable as *trpEG* but which contain numerous frameshifts and stop codons preventing the synthesis of an intact protein.) A comparison of the sequences between the 3.2 kb fragments with and without pseudogenes indicated 244 differences of which 93% were localized in an approximately 900-bp DNA segment which included the putative promoter and the N-terminus of *trpE* (Fig. 7a). These changes should result in the reduction or elimination of mRNA synthesis; if messenger is made, it would be translated into short peptides because of the numerous frame shifts and stop codons in the region of the N-terminus of *trpE*.

Another instance of *trpEG* silencing is illustrated by pTrpEG-Usn (Fig. 7b). This plasmid consists of a 3.2 kb unit of *trpEG* followed by 10–14 2.1 kb units consisting of DNA with a deletion of about 56% of the N-terminal region of *trpE* and an intact trpG. A more remarkable example of gene silencing is found in pTrpEG-R-Ps (Fig. 7c), which consists of a 3.6–3.8 kb unit containing *repAC2-trpEG* followed by 1.8 kb units consisting of *repAC1*, a deletion of *trpE*, and an intact *trpG* (van Ham et al., 1999). Preceding the latter is a short DNA segment which appears to be a remnant of DNA encoding the C-terminus of *trpE*. The structure of this plasmid suggests that, in *Buchnera* (Ps) of the family Pemphigidae, *trpEG* amplification had an origin independent of that of the remaining plasmids shown in Fig. 5. The initial plasmid probably contained tandem repeats of *repAC-trpEG*. Subsequently there was selection pressure for gene silencing resulting in a plasmid with one intact copy of *repAC-trpEG* and tandem repeats of *repAC-trpG* which contain a deletion of the putative promoter region and most of *trpE*.

Speculation Concerning trpEG Amplification

The following is a summary of the results obtained from studies of *trpEG* in *Buchnera*. 1) *trpEG* amplification is widespread in *Buchnera* within the family Aphididae and is also present in at least two members of the family Pemphigidae. In *Buchnera* from two aphid species of the latter family, *trpEG* is chromosomal and is found in the same location. In most cases *trpEG* amplification is affected by plasmids consisting of tandem repeats of the same or similar unit.

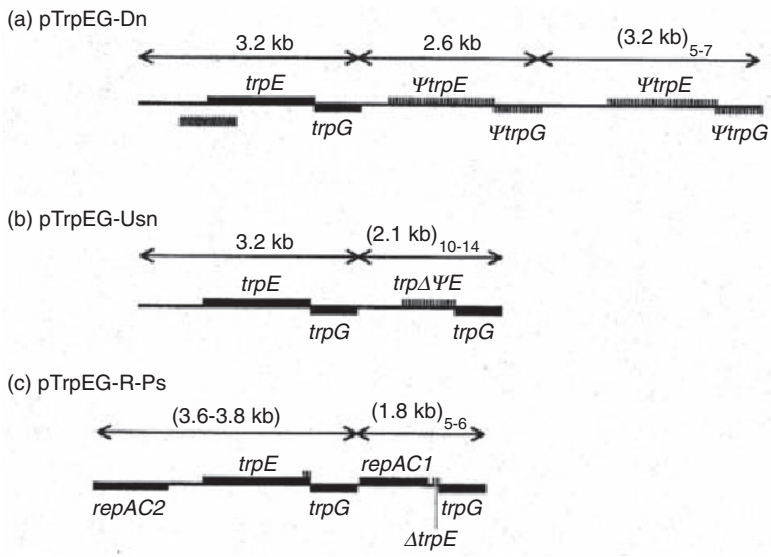

Fig. 7. Genetic map of plasmids which contain silenced trpEG. Stippled line in (a), region which in the pseudogene containing fragments has most of the changes; striped line in (a) and (b), pseudogenes (Ψ); Δ in (b), deletion of the N-terminal portion of trpE; striped line in (c), sequence homologous to the end of trpE. For references see text.

2) Evolution of plasmid-associated *trpEG* is vertical, that is, *Buchnera* from different aphid species do not exchange plasmids. 3) The *trpEG*-containing plasmids constitute at least two replicon types, one of which is based on the presence of DnaA boxes, while the other is based on the presence of interons and replication-initiation proteins related to plasmids of the IncA/C group. *Buchnera* from the related aphids Rm and Rp which belong to the first replicon type also have a gene and/or the remnant of a gene for a replication-initiation protein related to plasmids of the IncA/C group. 4) In *trpEG* plasmids of both replicon types, gene silencing of some of the *trpEG* tandem repeats is observed. 5) In *Buchnera* (Sg) the sequence of two of the 3.6-kb units is virtually identical, as is the sequence of the 2.6 and 3.2-kb *trpEG* pseudogene-containing units of *Buchnera* (Dn). While results above are derived from genetic analyses, the interpretations that follow are speculative and based in large part on analogies with other prokaryotic systems.

Gene amplification is currently viewed as a reversible aspect of genome plasticity which occurs at a frequency considerably higher than that of mutation in structural genes (Romero and Palacios, 1997; Roth et al., 1996). Gene amplification is frequently used by an organism as a means of increasing the amount of a growth-limiting enzyme to levels beyond that achieved by gene regulation of expression. One of the functions of *Buchnera* is the synthesis of essential amino acids (including tryptophan) for the aphid host. In almost all prokaryotes, the limiting enzyme TrpEG (which is feedback inhibited by tryptophan) regulates the activity of the tryptophan biosynthetic pathway. The *Buchnera* enzyme is probably also feedback inhibited by tryptophan since *trpE* has the conserved amino acid residues that are involved in feedback inhibition (Lai et al., 1994). To overproduce tryptophan, the activity of TrpEG must be increased, the potential effect of tryptophan accumulation on activity must be overcome, or both. Since even in high concentrations of tryptophan the activity of TrpEG is not fully inhibited, an increase in enzyme protein will result in increased tryptophan production. This has been the case in experiments on overproduction of tryptophan for industrial purposes; an increase in allosterically inhibitable TrpEG is the primary means of achieving excretion of high amounts of tryptophan into the medium (Katsumata and Ikeda, 1993).

In free-living bacteria, gene amplification is frequently transient. Its persistence depends on a constant selective pressure, the absence of which leads to a rapid decrease in number of repeats primarily by means of RecA-mediated homologous recombination (Roth et al., 1996). There may be differences or fluctuations in the levels of tryptophan in aphid diets; furthermore, the high level of TrpEG protein may impose an energy burden on the endosymbiont. Both of these conditions could provide short-term selective pressure for the elimination of plasmids from *Buchnera* (Baumann et al., 1997a). Consequently some mechanism of stabilization may be necessary for the maintenance of *trpEG*-containing plasmids. The gene *recA* has been cloned and sequenced from many bacterial taxa, and adequate oligonucleotide primers are available for its amplification by PCR (Eisen, 1995). We have made extensive unsuccessful attempts to detect this gene by PCR, suggesting that it may be absent from *Buchnera* or is greatly modified. Once plasmid stabilization occurs, the aphid may encounter conditions in which *trpEG* amplification is no longer necessary due to availability of tryptophan in the diet. If the usual mechanisms (homologous recombination?) which effect a decrease in *trpEG* amplification are absent, then one way of reducing the potentially wasteful synthesis of TrpEG is gene silencing.

In the past we have speculated that *trpEG* amplification is a property of rapidly growing aphids and that gene silencing may occur when following plasmid stabilization, the diet of aphids is nutritionally enriched (Baumann et al., 1997a; Lai et al., 1994, 1995, 1996). Recent studies have indicated that these speculations are overly simplistic. It has been suggested that the lack of *trpEG* amplification in *Buchnera* (Sc, Mr) which are in the Pemphigidae is due to the slow growth rate of these aphids compared to that of aphids within the Aphididae (Lai et al., 1995). There are, however, few studies on the growth rate of aphids, and the finding of amplification in *Buchnera* (Ps, Tc) which are in the family Pemphigidae and presumably also have a slow growth rate makes this explanation questionable. Dn causes major tissue histolysis of plants and it has been suggested that the presence of higher amounts of tryptophan in the diet may be the explanation for gene silencing (Lai et al., 1996). Recent studies on amino acid composition of ingested phloem sap are not strongly supportive of this hypothesis as a sole explanation for the presence of pseudogenes (Sandström and Moran, 1999; Sandström et al., 2000; Telang et al., 1999). *Buchnera* (Rp) and *Buchnera* (Sg) are similar in that both have pTrpEG consisting of four tandem repeats of a 3.6-kb unit (Fig. 5a). In spite of this similarity, Rp causes essentially no modification of the amino acid composition of plant phloem while Sg causes substantial increases (Sandström et al., 2000). Dn contains a plasmid with *trpEG* pseudogenes (Fig. 7a). The ingested diet of Dn has approximately doubled

concentrations of tryptophan, yet the changes it causes in the amino acid composition of phloem are less than those caused by Sg. This suggests that pseudogene formation is not solely the result of increased dietary tryptophan (Sandström et al., 2000; Telang et al., 1999). However, in the case of Usn, the presence of *trpEG* pseudogenes is consistent with the finding of unusually high levels of essential amino acids in the phloem diet (Sandström and Moran, 2000). Thus the availability of nutrients in plant sap may be a partial explanation for *trpEG* amplification and pseudogene formation, but other factors must also be involved.

In this connection it should be mentioned that the past speculations attempt to correlate adaptations of *Buchnera* with properties of the aphid host (growth rate, modification of nutrient content of plant sap). The environment of the endosymbiont is the bacteriocyte vesicle, which harbors the endosymbiont. This environment is a reflection of the activities of the aphid host and is a function of its ability to obtain nutrients from the plant as well as its demands on the biosynthetic attributes of the endosymbiont. Therefore, host properties, such as the efficiency of nutrient uptake from the plant and their transformation and delivery, may determine the nutritional parameters within the bacteriocyte vesicles and impose the selective pressure resulting in *Buchnera* adaptation to the endosymbiotic association.

Some of the phenomena encountered in pTrpEG from *Buchnera* also have been found in other systems. Promoter inactivation by multiple sequence changes is the mechanism used for silencing the expression of the *Bordetella pertussis* toxin gene (Gross and Rappuoli, 1988) and the expression of the *Bordetella* urease gene cluster (McMillan et al., 1998). The changes resemble those observed in gene silencing of *trpEG* in *Buchnera* (Dn; Fig. 7a). Multiple copies of nearly identical chromosomal enzyme-encoding genes have been found in *Thiobacillus ferroxidans* (Kusano et al., 1991) and *Nitrosospira* sp. (Norton et al., 1996). This situation is similar to that found with the repeats of *trpEG* and *trpEG* pseudogenes and has led to the postulation of mechanisms for the preservation of sequence identity of the repeated units (Klotz and Norton, 1998).

The phylogenetic trees constructed on the basis of plasmid-associated genes are congruent with the phylogenetic trees based on *Buchnera* chromosomal genes (Fig. 2a–c). One exception, the basal position of *Buchnera* (Tc; Fig. 2c), is probably an artifact arising from the more rapid change of *trpE* in this lineage (van Ham et al., 1999). These results strongly suggest that there is no exchange of *trpEG*-containing plasmids between endosymbionts of different aphids. Plasmids of the pTrpEG type (Fig. 5a–h) could have an endogenous origin. DnaA boxes are found in other locations of the *Buchnera* genome (Clark et al., 1998c), and their assembly with *trpEG* could generate a separate replicon. In contrast to these plasmids, the *repAC* genes of pTrpEG-R are related to replication initiation proteins of IncA/C plasmids. Thus, this plasmid may be the result of an invasion of *Buchnera* by an exogenous plasmid that recombined with endosymbiont genes, resulting in their amplification. It has been established that some bacteria may persist for a long time in insects; conceivably, such organisms transferred their plasmids to *Buchnera* during the infection of embryos or eggs at a stage at which the endosymbionts are not sequestered within bacteriocytes. It is, however, difficult to explain the presence of *repAC* and its remnant in *Buchnera* (Rm, Rp). One possibility is that it is the result of another invasion by a similar plasmid. However, organization of *ori-3.6* in *Buchnera* (Rm, Rp) closely resembles that of *Buchnera* of related aphids, suggesting a common plasmid origin for this group (Fig. 5a–c). Alternatively, van Ham et al. (1999) suggested that a plasmid of the pTrpEG-R type is the ancestor of all *trpEG* amplification plasmids. This hypothesis requires the subsequent occurrence of multiple losses of *repAC* and the interons in pTrpEG-R type plasmids and the acquisition of DnaA boxes in *Buchnera* of Aphididae.

pLeu Plasmids Figure 8 is an outline of the pathway of branched-chain amino acid biosynthesis. The gene for aspartokinase (*thrA*) has been found in *Buchnera* (Sg). The genes *ilvIH*, *ilvC*, and *ilvD* have been found in *Buchnera* (Sg, Dn, Sc, Mr) and encode three enzymes which func-

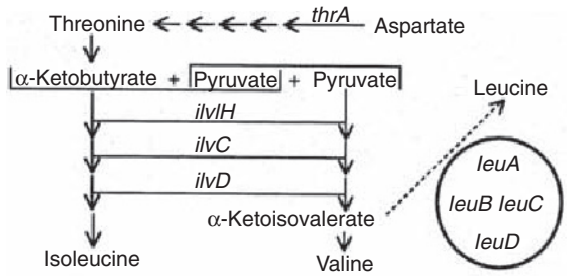

Fig. 8. Outline of the pathway for branched chain amino acid biosynthesis. Arrows, single enzymatic reactions; horizontal lines, enzymatic activities functional in both isoleucine and valine biosynthesis; dashed arrow, four enzymatic reactions; circle, plasmid containing genes for leucine biosynthesis. thrA was detected in *Buchnera* (Sg); ilvIH, ilvC, ilvD were detected in *Buchnera* (Sg, Dn, Sc, Rm); leuACBD were detected in *Buchnera* (Sg, Dn, Rm). For a description of genes see Table 4; for references see text.

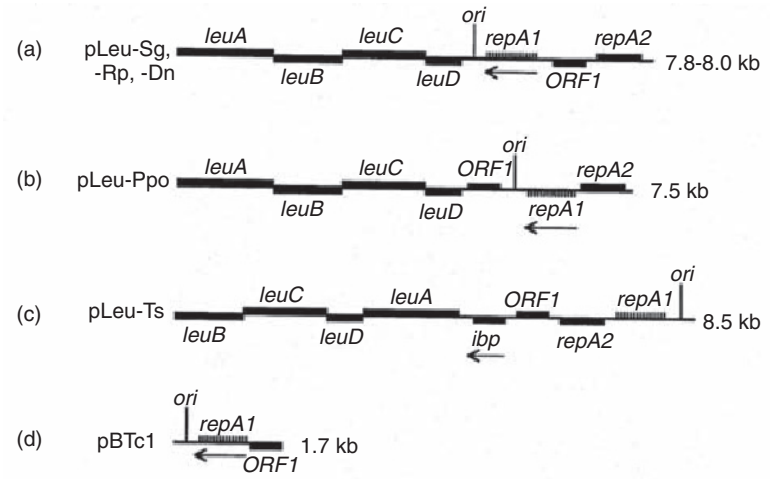

Fig. 9. Genetic maps of pLeu plasmids. Unless indicated by an arrow, transcription is left to right. ori, putative origin of replication; striped line, repA1 downstream of which is ori. For a description of genes see Table 4; for references see text.

tion in both the isoleucine and valine biosynthetic pathways (Clark et al., 1998c, 1999b; Thao and Baumann, 1998). The pathway of leucine biosynthesis is a branch off the valine pathway (Fig. 8). Bracho et al. (1995) found that in *Buchnera* (Rp), the genes for leucine biosynthesis (*leuABCD*) were present on a plasmid (Fig. 9a). This plasmid represents a third type (Table 7), designated pLeu, which is characterized by the presence of genes (*repA1*, *repA2*) encoding putative replication initiation proteins related to those of plasmids of the IncFII incompatibility group. Besides these genes, pLeu also contains *ORF1* encoding a putative membrane-associated protein. The closely related *Buchnera* (Sg, Rp, Dn) all contain very similar plasmids of 7.8 to 8.0 kb in which the genes are arranged in the same order (Fig. 9a; Baumann et al., 1999a; Bracho et al., 1995). In *Buchnera* from the more distantly related pLeu-Ppo (Fig. 2e), there are rearrangements of the *repA* genes and *ORF1* (Fig. 9b; Silva et al., 1998). All of these aphids are within the family Aphididae. In pLeu-Ts (pBTs1; Fig. 9c), which is from an aphid of the family Thelaxidae, there is also a rearrangement of the *leu* genes as well as an acquisition of *ibp* which encodes a heat shock protein (van Ham et al., 1997). All of these plasmids have a conserved region, downstream of *repA1*, which is probably an origin of replication (*ori*) (Baumann et al., 1999; Bracho et al., 1995; van Ham et al., 1997). Remarkably pBTc1 (Fig. 9d), a 1.7 kb plasmid from *Buchnera* (Tc), which is found in an aphid belonging to the family Pemphigidae, contains only *ori*, *repA1*, and *ORF1* and probably constitutes a minimal replicon.

In *Buchnera* (Sg), there are about 24 copies of pLeu per endosymbiont genome while in *Buchnera* (Dn) there are only 2 copies (Thao et al., 1998). This difference in functional gene copy number parallels that observed with pTrpEG in the endosymbionts of these two aphid species. In the case of pLeu, in which only one copy of the genes is present, the reduction in amplification in *Buchnera* (Dn) is achieved by means of a reduction of copy number. In pTrpEG, which contains tandem repeats of the same unit, the reduction in amplification is accomplished by means of pseudogene formation (Fig. 7a).

The similarities of pLeu plasmids suggest a single origin with pBTc1 (Fig. 9d) being the ancestral state (Baumann et al., 1999). In the lineage common to the Aphididae and the Thelaxidae, there was probably a duplication of *repA1* and the acquisition of *leu* genes. This was followed by a rearrangement of the genes and in one lineage the acquisition of *ibp* [for another interpretation, see van Ham et al. (1997)]. Silva et al. (1998) sequenced *repA2* from *Buchnera* of six additional aphids. A phylogeny based on this gene (Fig. 2e) as well as a more limited analysis based on *leu* genes (Fig. 2d) is congruent with trees established on the basis of other *Buchnera* chromosomal and plasmid genes. These results indicate that the pLeu plasmids are not exchanged among endosymbionts from different aphid species and that their evolution is vertical, as is the case with the *trpEG*-containing plasmids.

Unanswered Questions; Other Possible Adaptations One unanswered question is, why are only the *trp* and *leu* genes amplified? The endosymbiont produces other essential amino acids for the aphid host and their overproduction would in principle also be enhanced by plasmid amplification. It has been speculated that aphids make indole acetic acid which is involved in gall formation (Forrest, 1987). In many plant pathogens that cause gall formation, tryptophan is the

precursor of indole acetic acid (Patten and Glick, 1996). Amplification of *trpEG* is, however, found in aphids that do not produce galls (Sg, Dn, Rp, Usn) and is absent in some that do (Sc, Mr). Consequently this does not appear to be a probable explanation for *trpEG* amplification. Leucine, lysine, valine, arginine and threonine are the most common amino acid in aphids (Sandström and Moran, 1999), yet only genes for leucine biosynthesis have been detected on plasmids. It is possible that, in other cases where amino acids are overproduced for the host, an increase in enzyme activity is obtained by increasing the expression of the gene(s) by promoter modification. Alternatively changes of the allosteric properties of regulated enzymes may allow retention of activity in the presence of endproducts.

A possible example of the latter is *cysE* of *Buchnera* (Sg; Lai and Baumann, 1992b). This gene encodes an enzyme of the biosynthetic pathway of cysteine, and its activity is regulated by cysteine feedback inhibition (Kreditch, 1996). It has been established that the amino acids at the C-terminus of the *E. coli* enzyme are involved in cysteine feedback inhibition (Denk and Böck, 1987). The *Buchnera* (Sg) enzyme lacks these C-terminal amino acids and consequently is probably not subject to feedback inhibition by the endproduct; this change would result in cysteine overproduction (Lai et al., 1992b).

Secondary Endosymbionts of Aphids

Besides *Buchnera*, many aphids have additional endosymbionts usually called secondary (S-) endosymbionts (Buchner, 1965; Houk and Griffiths, 1980; Moran and Baumann, 1994). In many cases these endosymbionts are spheres or rod-shaped with different width and length; they were initially recognized by differences in size and shape from the round or oval *Buchnera*. The S-endosymbionts are also maternally inherited. They have not been extensively studied, and most of the available information is for the S-endosymbionts of the aphid Ap. Electron microcopic studies have shown that the rod-shaped S-endosymbionts are located within vesicles found in the flattened, syncytial, sheath cells which surround the bacteriome (Griffiths and Beck, 1973). Using a probe derived from *E. coli* 16S rDNA and restriction enzyme and Southern blot analysis of total aphid DNA, it was found that the S-endosymbiont from Ap contained a single copy of the 16S rDNA-gene (Unterman et al., 1989). DNA obtained from dissected bacteriocytes gave the same restriction pattern, indicating that the S-endosymbiont was located in the bacteriome. Two DNA fragments of 2.3 kb each were cloned and the 16S rDNA sequence determined. A phylogenetic analysis indicated that the S-endosymbiont was a member of the Enterobacteriaceae (Fig. 1). As in other members of this family, the 16S-rDNA gene of the S-endosymbiont was directly upstream of 23S rDNA (Unterman and Baumann, 1990).

Chen and Purcell (1997) found that 88% of the strains of Ap had the S-endosymbiont. In addition, the S-endosymbionts of Ap and *Macrosiphum rosae* were identical, suggesting recent infection or horizontal transmission. Interestingly, it was also found that the hemolymph of 48% of Ap strains contained a rod-shaped organism which had a 16S rDNA sequence nearly identical to that of *Rickettsia bellii*, an organism found in ticks (Chen et al., 1996). As a result of these studies strains of Ap became available which had 1) only the S-endosymbiont, 2) only the *Rickettsia* sp., as well as 3) neither of these two organisms. Chen (1996) injected one or both of these organisms into Ap, which originally lacked both, and observed their effect on fecundity, longevity and the length of the reproductive period. The results were complex in that they were affected by the plant on which the aphids grew and by the temperature of growth. At 20°C both the S-endosymbiont and the *Rickettsia* sp. reduced the fecundity, longevity and reproductive period of Ap on clover but had no significant effects on Ap grown on alfalfa or sweet pea (Chen, 1996). In some cases, both of these organisms appeared to cause an increase in the fitness of Ap when grown at 25°C. These results suggest that both the S-endosymbiont and the *Rickettsia* sp. can have either a deleterious or a beneficial effect on the host, depending on the environmental conditions (Chen, 1996). The S-endosymbiont, upon injection into the closely related species, *Acyrthosiphon kondoi*, was found to be pathogenic. Although the rate of maternal transmission of both the S-endosymbiont and the *Rickettsia* sp. was high, one instance of S-endosymbiont loss was observed (Chen and Purcell, 1997).

Studies based on light and electron microscopy have suggested that some S-endosymbionts in some aphid species may inhabit syncytial cells or, possibly, bacteriocytes that appear similar to those containing *Buchnera* (Buchner, 1965; Hinde, 1971b; Iaccarino and Tremblay, 1973). Fukatsu and Ishikawa (1993) surveyed 61 aphids for the presence of S-endosymbionts. Previously it was found that *Buchnera* (Ap) overproduced GroEL (Kakeda and Ishikawa, 1991). Using anti-*E. coli*-GroES for immunoprecipitation of

Buchnera (Ap) GroES, it was concluded that *Buchnera* produced low levels of this protein (Kakeda and Ishikawa, 1991). This antiserum as well as anti-*E. coli*-GroEL was used to detect GroEL and GroES in immunoblots of whole aphid extracts as well as for histochemical detection in thin sections of aphids. From these experiments it was concluded that the synthesis of substantial amounts of GroES by S-endosymbionts distinguishes them from *Buchnera*, and that this property can be used for the identification of S-endosymbionts (Fukatsu and Ishikawa, 1993). These studies have a number of problems that limit their general applicability to the survey of S-endosymbionts. The principal one is the use of antisera against *E. coli* GroES for the detection of cross reactivity of GroES from organisms that have an unknown relationship to *E. coli*. Since it is probable that many of the S-endosymbionts are members of the Enterobacteriaceae, a stronger cross-reaction would be expected with their proteins than with the proteins from *Buchnera*; consequently, an increased reactivity need not indicate a major difference in the amount of the protein. Conversely, in those cases where the S-endosymbiont is not a member of the Enterobacteriaceae, the distant relationship may preclude a strong cross-reaction (Eremeeva et al., 1998). The cross-reactivity of the *Buchnera* and S-endosymbiont proteins with the anti-*E. coli* protein antisera has not been compared. Finally, the relative production of GroES may vary among S-endosymbionts of different types.

The studies of Fukatsu and Ishikawa (1993, 1998), in which thin sections of aphids were stained by immunohistochemistry and examined by light microscopy, do suggest that in many aphids the S-endosymbionts occupy bacteriocytes distinct from those containing *Buchnera*. The authors also state that the S-endosymbionts have a variety of different shapes. The methods used and the photographs presented do not, however, allow adequate visualization of cell shape and the resolution of bacteriocyte structure. In addition the designation of some of the endosymbionts as *Buchnera* or S-endosymbionts appears to be arbitrary.

Fukatsu et al. (1998) used group-specific oligonucleotide probes for *in situ* detection of aphid P- and S-endosymbionts. A universal eubacterial 16S rRNA probe was used as well as probes specific for the 23S rRNA γ- and β-subdivision Proteobacteria. The sequences of the latter two probes differ by only one nucleotide. *Buchnera* and S-endosymbionts all hybridized with the 16S eubacterial probe, although the intensities of the signal differed considerably. Curiously the putative P-endosymbionts of two out of seven aphids did not hybridize with the 23S γ-subdivision probe. Using the total DNA preparation from these aphids, the 16S rDNA was amplified, cloned and sequenced. Two sequences were detected in each aphid DNA preparation, and one of these was related to *Buchnera* 16S rDNA. Based on this result, it was concluded that these aphids contained *Buchnera* but that their 23S-rDNA gene was changed to such an extent that hybridization with the γ-subdivision probe no longer occurred. This conclusion is questionable since the 23S rDNA of *Buchnera* (Sg, Dn, Sc, Mr), which span the diversity of aphid hosts (Fig. 2), contains the exact sequence complementary to the probe used (Clark et al., 1999b; Rouhbakhsh and Baumann, 1995). The S-endosymbiont of *Tetraneura radicicola* hybridized with the probe to the β-subdivision but not to the γ-subdivision, suggesting that this endosymbiont is a member of the former group. Since there is only a single nucleotide difference between these two probes, a confirmation of this conclusion by sequencing the rDNA from this organism seems desirable. The S-endosymbiont from two other aphid species did not hybridize with either the γ- or β-subdivision probe. In view of the technical difficulties encountered with some of the specimens, these conclusions cannot be interpreted as indicating that the S-endosymbionts of these aphids belong to different bacterial groups.

Absence of a Stable Bacterial Flora in Aphid Guts

Aphids maintained under clean conditions do not appear to have a bacterial gut flora (Douglas, 1990; Grenier et al., 1994; Harada and Ishikawa, 1993). Older aphids as well as aphids reared under crowded conditions may acquire a gut flora which appears to consist of members of the Enterobacteriaceae (*Serratia*, *Erwinia*), *Pseudomonas*, *Staphylococcus*, and *Bacillus* (Grenier et al., 1994; Harada and Ishikawa, 1993). These organisms are frequently associated with plant surfaces. None of these organisms has a close relationship to *Buchnera*, precluding a recent common ancestor from which they and *Buchnera* are descended. The presence of this bacterial flora has a deleterious effect on aphid performance (Grenier et al., 1994). The actual bacterial numbers have not been established. Harada et al. (1996) have isolated 38 bacterial strains from the guts of 20 aphids. This hardly suggests the presence of an indigenous bacterial flora and the numbers are insignificant compared to the numbers of *Buchnera* or the S-endosymbionts. These studies indicate that the guts of aphids are generally sterile but under certain conditions a transient bacterial gut flora may be present.

Endosymbionts of Other Plant Sap-Utilizing Insects

Psyllids, whiteflies and mealybugs are three separate lineages of the suborder Sternorrhyncha and contain P-endosymbionts from three distinct bacterial groups (Table 1, Fig. 1). These insects have obligatory sexual reproduction with the young hatching from eggs (Borror et al., 1989). The endosymbionts are housed within bacteriocytes, and at least during some stage of the insect's life cycle, the bacteriocytes are associated with the ovarioles resulting in the transmission of endosymbionts to the eggs (Buchner, 1965).

Psyllid Endosymbionts

Fukatsu and Nikoh (1998) sequenced the 16S rDNA of endosymbionts from *Anomoneura mori*, while Spaulding and von Dohlen (1998) performed a similar study of the endosymbionts of *Blastopsylla occidentalis*, *Pachypsylla venusta*, and *Trioza magnoliae*. These authors came to the same conclusion, namely that the P-endosymbionts of psyllids constitute a distinct lineage within the γ-subdivision of the Proteobacteria (Fig. 1). These endosymbionts have an unusual property, namely, the lowest known G+C content of any 16S rDNA (36.4 mol%). In addition, this lineage appeared to have a substantial acceleration of the rate of evolutionary change within the 16S sequence. *A. mori*, *B. occidentalis*, and *T. magnoliae* all had different S-endosymbionts, which belong in the γ-subdivision. Only the P-endosymbiont was found in *P. venusta* (Spaulding and von Dohlen, 1998). The studies were limited to few taxa but were consistent with cospeciation of the P-endosymbiont and the psyllid host and multiple acquisitions of S-endosymbionts (Fukatsu and Nikoh, 1998; Spaulding and von Dohlen, 1998).

Psyllids contain bilobed bacteriomes made up of round uninucleate bacteriocytes and a multinucleate syncytial region (Buchner, 1965). Many psyllids have endosymbionts in both the bacteriocytes and the syncytium; some have endosymbionts only within the bacteriocytes (Buchner, 1965). Using electron microscopy, Chang and Musgrave (1969) and Waku and Endo (1987) found that two psyllid species have endosymbionts in the bacteriocytes which are distinguishable from those found in the syncytium and that both endosymbiont types have a Gram-negative cell wall. In general, the bacteriocyte-associated endosymbionts are more numerous than the syncytium-associated endosymbionts (Buchner, 1965). Fukatsu and Nikoh (1998), using an oligonucleotide probe specific for the P- or S-endosymbiont, showed by means of *in situ* hybridization that the former was localized in the bacteriocytes while the latter was in the syncytium (Fig. 10). There is currently no information on the requirement of the endosymbiont(s) by the psyllid host or on their function. The similarity in diet between psyllids and aphids raises the possibility that psyllid endosymbionts may provide nutrients as do *Buchnera*.

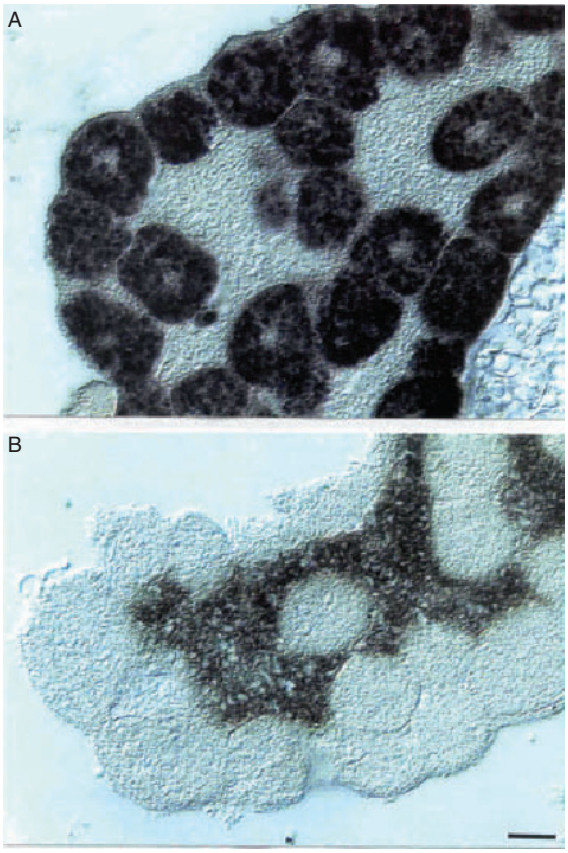

Fig. 10. Light micrographs of a bacteriome of the psyllid Anomoneura mori. (a) In situ hybridization using an oligonucleotide probe specific for the P-endosymbiont 16S rRNA, which reacts with bacteriocytes containing these endosymbionts. (b) In situ hybridization using an oligonucleotide probe specific for the 16S rRNA of the S-endosymbiont of this psyllid, which reacts with the endosymbiont located in the syncytium. Bar = 20 μm. From Fukatsu and Nikoh (1998) with permission from the authors and ASM Press.

Whitefly Endosymbionts

16S rDNA sequences have been obtained for endosymbionts of *Bemisia tabaci*, *B. argentifolii* (previously *B. tabaci* B biotype), *Siphonius phillyreae*, and *Trialeurodes vaporariorum* (Clark et al., 1992). The P-endosymbionts of these insects are a lineage within the γ-subdivision of the Proteobacteria (Fig. 1). *B. tabaci* and *B. argentifolii* have an S-endosymbiont which is

a member of the Enterobacteriaceae. The P-endosymbionts and the S-endosymbionts from these two species have identical 16S rDNA sequences, consistent with their close relationship (Brown et al., 1995; Clark et al., 1992).

The ultrastructure of the endosymbionts of *B. tabaci*, *B. argentifolii*, and *T. vaporariorum* has been studied by electron microcopy (Costa et al., 1993b, 1995). There is evidence for at least two morphological types. Whiteflies are unusual in that they transmit an entire bacteriocyte containing endosymbionts to the egg (Buchner, 1965; Costa et al., 1996). Some prokaryote-specific antibiotics affect the growth and development of whiteflies, indicating a requirement for the endosymbiont(s) by the host (Costa et al., 1993a, 1997).

Mealybug Endosymbionts

16S rDNA sequences have been obtained for endosymbionts of *Pseudococcus longispinus*, *P. maritimus*, and *Dysmicoccus neobrevipes* (Munson et al., 1992), and these organisms were found to be a distinct lineage within the β-subdivision of the Proteobacteria (Fig. 1). The morphology of endosymbionts from several mealybug species has been studied by means of electron microscopy (Tremblay, 1989). Within the bacteriocytes the endosymbionts appear to be embedded in mucous spherules of unknown composition. There is no information on the function or the requirement for the endosymbionts by the host.

Tsetse Fly Endosymbionts

Tsetse flies (genus *Glossina*) are important vectors of trypanosomes, which are causative agents of African sleeping sickness and various diseases of animals (Harwood and James, 1979). They have a somewhat unusual reproductive cycle in that the female gives birth to fully grown larvae. Only one larva is carried at a time within the uterus. During this stage the larva is fed nutritive fluids from special glands, commonly known as "milk glands." The female requires several blood meals to complete the development period of each larva, and it is these blood meals which result in the transmission of trypanosomes (Harwood and James, 1979).

Tsetse may be associated with three prokaryotes: 1) *Wigglesworthia* (P-endosymbionts), 2) *Sodalis* (S-endosymbionts), and 3) *Wolbachia*. The last are parasites found in reproductive tissue and causing reproductive disorders (O'Neill et al., 1997) and will not be considered here.

Table 8. Species of tsetse (*Glossina*) for which the 16S rDNA sequence of *Wigglesworthia* has been determined.[a]

G. austeni
G. brevipalpis
G. fuscipes
G. morsitans centralis
G. m. morsitans
G. palpalis gambiensis
G. p. palpalis
G. tachinoides

[a]Chen et al. (1999).

Wigglesworthia—the Primary Endosymbiont of Tsetse Flies

Phylogeny Based on 16S rDNA, *Wigglesworthia* was found to constitute a distinct lineage within the γ-3 subgroup of the Proteobacteria (Fig. 1; Aksoy et al., 1995; Chen et al., 1999). These organisms are related to but distinct from *Buchnera* of aphids and the P-endosymbionts of carpenter ants (Fig. 1). Using the host rDNA transcribed spacer-2, it was found that the phylogeny of the host was the same as that of *Wigglesworthia*, indicating cospeciation of the host and the endosymbiont (Chen et al., 1999). These results suggest a single infection of a tsetse ancestor with a bacterium followed by long-term vertical transmission of the endosymbiont, that is, a lack of exchange of *Wigglesworthia* between different tsetse fly species. The age of this association has been estimated to be at least 50 million years (Aksoy et al., 1997). A list of the species of tsetse flies for which the 16S rDNA of *Wigglesworthia* has been sequenced is presented in Table 8.

Taxonomy The genus *Wigglesworthia* contains one species, *W. glossinidia*, which designates the lineage consisting of the P-endosymbionts of tsetse flies (Aksoy, 1995b). The type strain of this species is the P-endosymbiont of *G. morsitans morsitans*.

Habitat Tsetse flies contain a U-shaped bacteriome located in the anterior region of the gut, which is made up of bacteriocytes containing *Wigglesworthia* (Aksoy, 1995b; Aksoy et al., 1995). These endosymbionts have a Gram-negative cell wall and are somewhat pleomorphic, occurring mostly as 4–5 m long rods. They are found free (not enclosed within host-derived vesicles) in the bacteriocyte cytoplasm. *Wigglesworthia* is maternally transmitted. Since neither the milk gland nor the developing eggs contain *Wigglesworthia*, the mechanism of their transmission is not known (Aksoy et al., 1997).

Physiology The feeding of tsetse flies on animals immunized with *Wigglesworthia* results in

elimination of the P-endosymbiont and sterility of the flies (Nogge, 1976). A similar effect is observed upon treatment of tsetse with prokaryote-specific antibiotics (Aksoy et al., 1995; Nogge, 1976, 1982). These results indicate that the P-endosymbiont is essential for reproduction. There is evidence that one of the functions of the *Wigglesworthia* is the production of B-complex vitamins (Nogge, 1982). *Wigglesworthia* produces a high level of GroEL (Aksoy, 1995a). In this respect it is similar to a number of other endosymbionts as well as other intracellular organisms (Hogenhout et al., 1998).

Genetics The *Wigglesworthia* genome has one copy of the 16S rRNA gene (Aksoy, 1995a). In this organism 16S-rRNA gene is directly upstream of 23S-rRNA gene, suggesting that, as in the case of many other bacteria, these genes are a part of a single transcription unit. The presence of one copy of the rRNA operon is characteristic of slow-growing bacteria and also is found in several other endosymbionts (Baumann et al., 1995).

Sodalis—The Secondary Endosymbiont of Tsetse Flies

Tsetse flies may also contain S-endosymbionts. These are primarily found within midgut cells but also have been detected in hemolymph and in a variety of other tissues excluding ovaries (Aksoy et al., 1997; Beard et al., 1993b; Cheng and Aksoy, 1999). Their numbers are age-dependent, being higher in older insects (Cheng and Aksoy, 1999). The 16S rDNA has been sequenced from the S-endosymbionts of five different tsetse fly species, and it was found that they are members of the Enterobacteriaceae (Aksoy et al., 1997; Beard et al., 1993b). The sequences were found to be virtually identical, indicating either multiple recent infections with the same organism or horizontal transmission of the S-endosymbiont. The S-endosymbiont is maternally transmitted via the "milk gland" secretions to developing larvae (Aksoy et al., 1997).

The S-endosymbionts have been cultivated in cell-free liquid media (Beard et al., 1993b) and recently on solid media (Dale and Maudlin, 1999). The latter allowed a phenotypic characterization of this organism and led to its assignment into a new genus and species, *Sodalis glossinidius* (Dale and Maudlin, 1999). This species consists of Gram-negative rods 1–1.5 m in diameter and 2–12 m in length. It is microaerophilic, lacking catalase, and has a relatively limited capacity for carbohydrate utilization.

The S-endosymbiont has seven copies of 16S rDNA, a number which is similar to that found in rapidly growing free-living organisms (Aksoy, 1995a). Plasmids of 80 kb and about 130 kb have been detected in these organisms (Beard et al., 1993b). The S-endosymbiont has been transformed with pSUP204, and plasmid-encoded resistance to ampicillin, tetracycline and chloramphenicol was expressed (Beard et al., 1993b). Similarly the S-endosymbiont has been transformed with a pSUP204 derivative, which expressed the green fluorescent protein, allowing ready visualization of this organism in insect tissues (Cheng and Aksoy, 1999).

In one case, the S-endosymbiont from one tsetse fly species when microinjected into another species became pathogenic, killing the flies within 48 h (Cheng and Aksoy, 1999). This result is similar to the observations made with the S-endosymbiont of aphids (Chen and Purcell, 1997). The variation in the number of S-endosymbionts and their possible absence from some insects suggest that they do not perform a function essential for the survival of tsetse flies.

Sitophilus (Weevils) Endosymbionts

Weevils of the genus *Sitophilus* are major pests of stored grain (Borror et al., 1989). The female bores a hole in kernels and deposits an egg. The larva develops inside the grain from which the young adults emerge. Three related species have been studied with respect to their endosymbionts, *S. oryzae*, *S. granarius*, and *S. zeamais* (Dasch et al., 1984). Of these three species, the most extensive studies deal with *S. oryzae* (Nardon and Grenier, 1988). In addition, weevils may harbor the pathogen *Wolbachia* (O'Neill et al., 1997).

Phylogeny Early studies of *Sitophilus* endosymbiont morphology and the G+C content of its DNA suggested that weevils have different endosymbionts (Dasch, 1975; Dasch et al., 1984; Grinyer and Musgrave, 1966; Musgrave and Grinyer, 1968). *S. oryzae* has one endosymbiont with a G+C content of about 54 mol%, *S. granarius* has one with a G+C content of 50 mol%, while *S. zeamays* appears to have both endosymbionts (Dasch, 1984; Heddi et al., 1998). One type of endosymbiont 16S-rDNA sequence was detected in *S. oryzae*, and two types were detected in *S. zeamais* (Campbell et al., 1992; Heddi et al., 1998). Phylogenetic analysis indicated that all of these endosymbionts

are members of the family Enterobacteriaceae (Fig. 1).

Habitat

In larvae, endosymbionts are present in bacteriocytes which make up a bacteriome located at the junction of the foregut and the midgut as well as in the rudimentary ovaries (Charles et al., 1995; Nardon and Grenier, 1988). The endosymbionts are transmitted via the eggs. *S. oryzae* endosymbionts are rod-shaped, 5–15 m long, and free (not within host-derived vesicles) in the cytoplasm (Dasch et al., 1984; Nardon and Grenier, 1988).

Physiology

S. oryzae may be cured of endosymbionts by treatment with heat or antibiotics (Baker and Lum, 1973; Nardon and Grenier, 1988). Such aposymbiotic weevils are softer and paler, have an increased development time, and the fertility of their eggs is reduced (Nardon and Gernier, 1988). On some nutrient-rich grains, they can grow indefinitely. Aposymbiotic weevils lack bacteriomes, indicating that the endosymbiont triggers their development. Naturally occurring aposymbiotic weevils also may be found. There is evidence that one of the functions of the endosymbiont is the synthesis of vitamins as well as possibly phenylalanine or tyrosine (Baker, 1975, 1979; Wicker and Nardon, 1982). An additional function is the conversion of excess methionine in the diet to methionine sulfoxide (Gasnier-Fauchet and Nardon, 1986, 1986). Aposymbiotic weevils also have mitochondria with reduced levels of enzymes involved in respiration (Heddi et al., 1991). Isolated endosymbionts of *S. oryzae* do not consume oxygen and lack a number of enzymes of respiratory metabolism (Heddi et al., 1991, 1993). These results suggest that they have an anaerobic metabolism. As in the case of *Buchnera* and *Wigglesworthia*, the endosymbionts of *S. oryzae* overproduce GroEL (Charles et al., 1997b). Unlike *Buchnera*, the endosymbionts of *S. oryzae* have a heat shock response, as is indicated by an increase of *groEL* mRNA (Charles et al., 1997b).

Genetics

The genome of the endosymbiont of *S. oryzae* is 3.0 Mb and has two copies of the rRNA operon (Charles et al., 1997a). In addition, the endosymbiont contains a plasmid of about 138 kb. This genome size puts the endosymbiont within the range of many free-living bacteria (Heddi et al., 1998).

Comparisons with Other Associations

Although the information is somewhat limited, comparison of the *Sitophilus*-endosymbiont association with other insect-endosymbiont associations suggests that the former has some unique features. In several insect endosymbiotic associations, the S-endosymbionts are members of the Enterobacteriaceae, suggesting that organisms within this lineage have an enhanced capacity to enter into such associations. An interesting feature of the *Sitophilus* association is that these organisms are the sole endosymbionts. The morphological diversity of the endosymbionts, the large endosymbiont genome size, and the fact that aposymbiotic weevils are viable suggest that the associations arose through multiple recent infections and that major adaptations resulting in obligatory mutual interdependence of both partners have not evolved as yet. This association may consequently be an example of an endosymbiosis at an early stage of its development.

Carpenter Ant Endosymbionts

Ants feed on complex diets, and the presence of endosymbionts has been reported in only two groups. These two groups are the genus *Formica* and the genus *Camponotus*, commonly known as the carpenter ants (Borror et al., 1989; Buchner, 1965; Dasch et al., 1984). Both groups can use a broad range of food types but typically utilize plant nectar and honeydew (the liquid feces of sap-feeding Homoptera) as major components of their diet. Only in the carpenter ants have symbionts been studied using modern methods, and we focus on these. The contribution of ant endosymbionts to host nutrition is not clear (Dasch et al., 1984).

Phylogeny

The G+C content of the DNA of carpenter ants is 30–32 mol% (Dasch, 1975; Dasch et al., 1984). The sequence of the 16S rDNA has been determined for endosymbionts of the species *Camponotus floridanus*, *C. rufipes*, *C. ligniperdus* and *C. herculeanus* (Schröder et al., 1996). A phylogenetic analysis indicated that the endosymbionts constitute a distinct, monophyletic group related to, but different from, the endosymbionts of aphids, tsetse flies, and the members of the Enterobacteriaceae (Fig. 1). The order of branching reflects the relationships between the carpenter ant species and is consistent with a single infection and subsequent vertical evolution of the endosymbionts. The age of the association is estimated at over 100 million years

(Schröder et al., 1996). In endosymbionts of carpenter ants, the 16S-rRNA gene is not directly upstream of the 23S-rRNA gene, suggesting that these genes are organized in two transcription units, as is the case in *Buchnera* (C. Sauer and R. Gross, personal communication).

Habitat

In both workers and queens of *Camponotus*, the endosymbionts are located in bacteriocytes, which are intercalated between epithelial cells of the midgut (Fig. 11a) (Buchner, 1965; Schröder et al., 1996). The endosymbionts are rods of 1 µm in width to 5–15 µm in length (Fig. 11b). They have a Gram-negative type cell wall and are free (not enclosed in host-derived vesicles) in the cytoplasm (Schröder et al., 1996). Transmission is via infection of the ovaries and incorporation into the eggs.

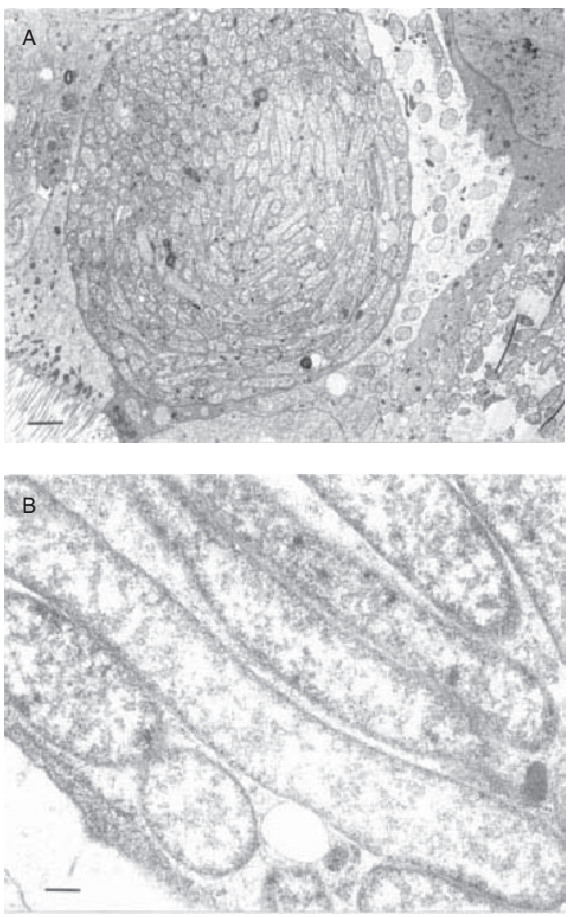

Fig. 11. Electron micrographs of the endosymbionts of the carpenter ant *Camponotus floridanus*. (a) Bacteriocyte containing the endosymbionts, bar= 3.0 µm; (b) ultrastructure of the endosymbionts showing the Gram-negative cell wall and the absence of a vesicular membrane, bar= 0.3 µm. Photos courtesy of C. Sauer and R. Gross.

Blattabacterium-Endosymbionts of Cockroaches and Termites

Cockroaches (order Blattaria) utilize a complex diet and harbor prokaryotic endosymbionts (Dasch et al., 1984). It has been hypothesized that cockroaches and termites (order Isoptera) are phylogenetically related (Kambhampati, 1995). Common ancestry is suggested from the fact that the wood-eating cockroach, *Cryptocercus punctulatus*, has a cellulose-digesting protozoal gut flora which is similar to that of termites (reviewed in Bandi and Sacchi, 1999). In addition, *Mastotermes darwiniensis*, a primitive termite, lays eggs in rows resembling those made by cockroaches (Borror et al., 1989; Sacchi et al., 1998b).

Phylogeny

The G+C content of the DNA of *Blattabacterium* is 26–28 mol% (Dasch, 1975; Dasch et al., 1984). Phylogenetic analysis of the 16S rDNA from *Blattabacterium* of cockroaches and the termite *M. darwiniensis* indicates that the endosymbionts form a distinct lineage within the *Flavobacter-Bacteroides* group of bacteria (Bandi et al., 1994, 1995; Fig. 1). The phylogenetic tree obtained on the basis of endosymbiont 16S rDNA is the same as that derived from host taxonomy. This result is consistent with a single infection in an ancestor of cockroaches and termites and vertical evolution of the endosymbionts, that is, a lack of endosymbiont exchange among different species. *M. darwiniensis* is the only termite known to harbor *Blattabacterium*, suggesting that, with the exception of this termite, the endosymbionts were eliminated in the lineage leading to the present termite species (Bandi and Sacchi, 1999; Bandi et al., 1997). The 16S-rDNA sequence has been determined for endosymbionts of the cockroach species *Periplaneta australasiae*, *P. americana*, *Blatella germanica*, *Pynoscelus surinamensis*, *Nauphoeta cinerea* and *C. punctulatus*, as well as the termite species *M. darwiniensis* (Bandi et al., 1994, 1995). The association between *Blattabacterium* and termites is estimated to be 135–300 million years old (Bandi et al., 1995).

Taxonomy

The genus *Blattabacterium* contains one species, *B. cuenoti*, and currently designates the lineage consisting of the bacteriocyte-associated endosymbionts of cockroaches and one termite (Bandi et al., 1995; Dasch et al., 1984). The type strain is the endosymbiont of *Blatta orientalis* (Dasch et al., 1984).

Habitat

Blattabacterium is found in bacteriocytes, polyploid cells which are found within the abdominal fat bodies of cockroaches and the termite *M. darwiniensis* (Fig. 12a) (Bigliardi et al., 1995; Sacchi et al., 1996, 1998a, b). The endosymbionts are rods of 1 μm in width and 1.6–9 μm in length (Fig. 12b; Dasch et al., 1984). They have a Gram-negative cell wall and are located within vesicles derived from the host cell. The bacteriocytes originate from plasmatocytes, which are phagocytic cells present in the hemolymph. There are extensive electron microscopic studies that document the infection and differentiation of plasmatocytes into bacteriocytes and the transmission of the endosymbionts to oocytes and eggs (Bigliardi et al., 1995; Sacchi et al., 1996, 1998a, b).

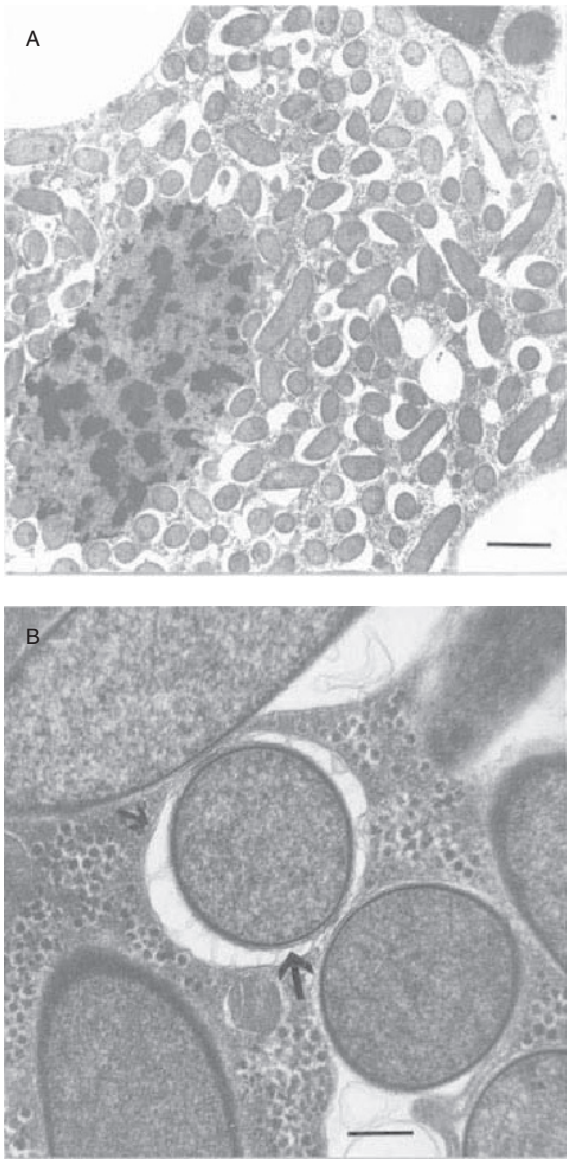

Fig. 12. Electron micrographs of *Blattabacterium cuenoti*, the endosymbiont of cockroaches and termites. (a) Endosymbiont within bacteriocytes of the cockroach *Periplaneta americana*, bar= 3.0 μm (photo courtesy of L. Sacchi); (b) ultrastructure of the endosymbiont of *Cryptocercus punctulatus* showing the Gram-negative cell wall (large arrow) and the vesicular membrane (small arrow), bar= 0.3 μm. From Sacchi et al. (1998) with permission of the authors and Balaban Publishers.

Physiology

Aposymbiotic cockroaches can be obtained by rearing the insects on antibiotic-containing foods (Bandi and Sacchi, 1999; Dasch et al., 1984). The resulting insects can be propagated on enriched diets. The aposymbiotic insects are smaller in size, light in color, and have a reduced fecundity and an increased development time. They also have an increased level of uric acid in fat bodies, suggesting that one function of *Blattabacterium* is nitrogen recycling (Cochoran, 1985). The proximity of bacteriocytes and urate cells (which store uric acid) in the fat body and the presence of adhesion sites between their plasma membranes suggest direct metabolic interactions between these cells (Sacchi et al., 1998a). There is also evidence that the bacteria provide essential amino acids for the host (Henry, 1962).

Isolation

P-endosymbionts have been isolated from aphids and also from *Sitophilus*. In the case of aphids, the starting material is usually whole insects; in the case of *Sitophilus*, the starting material is dissected bacteriomes. Since the two methods are similar, only the method for the isolation of *Buchnera* from aphids will be considered. Details of the isolation of the endosymbionts from *Sitophilus* are described by Heddi et al. (1991).

The methods for the isolation of *Buchnera* have been developed by Ishikawa (1982), Sasaki and Ishikawa (1995), and Charles and Ishikawa (1999). The resulting preparations are suitable for isolation of high molecular weight DNA for genome analysis as well as for physiological studies. The best criteria of purity have involved examination of the preparations by electron microscopy, which also allows determination of whether the endosymbionts are still within host-derived vesicles. Both the aphids and the endosymbionts have a similar mol% G+C in their DNA (Ishikawa, 1987; Unterman and Baumann, 1990). Consequently, endosymbiont DNA

cannot be separated from host DNA by CsCl density gradient centrifugation.

As starting material, it is best to use an aphid strain that has only *Buchnera* and lacks S-endosymbionts. All of the reagents and equipment are kept on ice, and the procedures are performed as rapidly as possible. Approximately 2 to 3 g (wet weight) aphids are transferred to a 1.5 cm diameter tissue grinder. Ten ml of buffer A of Ishikawa (1982) is added, and the aphids are ground with a lose fitting plunger for 5 min. [Buffer A contains 0.25 M sucrose, 35 mM Tris-HCl (pH 7.6), 25 mM KCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol.] The preparation is then passed through a double layer of a nylon mesh to remove large particulate material. The filtrate is brought to a volume of about 100 ml with buffer A and then quickly passed through a 100-µm nylon filter followed by filtration through 20-µm and 10-µm nylon filters (in some cases filtration through 5- and 3-µm filters is also performed). Only slight vacuum pressure is applied during the last two filtration steps. The suspension is centrifuged in a swinging bucket rotor for 6–10 min at 1,500 g. The pellets are gently resuspended in 1–2 ml buffer A and centrifuged through a Percoll gradient (12,000 g, 15 min). The gradient consists of 27–70% Percoll in buffer A, 5% PEG 6000, 1% Ficoll, and 1% bovine serum albumin (Pharmacia Biotech, Uppsala, Sweden; Charles and Ishikawa, 1999). Mitochondria are in the upper phase, host nuclei are in the pellet, and *Buchnera* appears as a green band.

Identification

Currently none of the P-endosymbionts has been cultured and consequently identification is based primarily on sequence analysis of their 16S rDNAs. Since these studies are just beginning, we will briefly discuss the methods used for both their characterization and identification. The success of the approach used in most of the studies has been dependent on the use of fresh or frozen insect samples which meet the following criteria: 1) the predominant bacterial flora of the insect consists of one or a few endosymbiont types, 2) there is no significant gut flora, and 3) the samples are relatively clean.

An ideal study would utilize the full-cycle rRNA analysis formulated by Amann et al. (1995). In this approach the 16S rDNA is amplified by PCR and sequenced. Based on comparisons with 16S rDNA(s) in databases, the organism(s) are identified. Specific oligonucleotide probes are designed and used in *in situ* hybridization to identify the endosymbiont associated with the sequence. This approach is extremely useful when more than one endosymbiont is present and, in the case of insects, has been applied to the identification of two types of psyllid endosymbionts (Fukatsu and Nikoh, 1998).

In studies in which the primary goal was a phylogenetic characterization of the P-endosymbiont, one approach used is an initial thorough study of one or more representative insects that can be cultivated or are readily available. Then characterization is extended to taxa obtained as field collections and preserved in dry ice or absolute ethanol. For example, in the initial study of aphid endosymbionts, the insect specimen chosen (Ap) was known from electron microscopy to harbor two morphologically distinct endosymbionts (Unterman et al., 1989). Using total aphid DNA, a probe to *E. coli* 16S rDNA, and restriction enzyme and Southern blot analysis, only two restriction maps could be constructed corresponding to the two endosymbionts. There were differences in the intensities of the bands, indicating that one of the endosymbionts was present in larger numbers than the other. In addition, the results were consistent with the presence of only one copy of the 16S-rRNA gene per endosymbiont genome. Based on the restriction maps, DNA fragments containing 16S-rRNA genes of both endosymbionts were cloned and sequenced. In addition, DNA was isolated from dissected bacteriomes, and restriction enzyme and Southern blot analysis indicated the association of both endosymbionts with the bacteriome. Subsequently, restriction enzyme and Southern blot analysis using DNA from other aphid species indicated the presence of only one copy of the 16S-rRNA gene, a finding consistent with only one or a single predominant endosymbiont (Munson et al., 1991b). Upon amplification of the 16S rDNA by PCR, the sequences detected were all related to *Buchnera*. This approach is suitable for the study of insects in which there is one predominant P-endosymbiont and does not exclude the possibility of not detecting S-endosymbionts that are present in lower numbers.

Instead of initial studies involving restriction enzyme and Southern blot analysis, it is much more convenient to use oligonucleotides complementary to the front and back of 16S rDNA and PCR to amplify DNA fragments for cloning, sequencing, or both. Localization of the endosymbiont may be performed by dissection of different tissues, purification of the DNA, and amplification of the 16S rDNA by PCR (Aksoy et al., 1995; Bandi et al., 1994, 1995; Schröder et al., 1996). Differences in the types of rDNA amplified may be established by restriction fragment polymorphism (Clark et al., 1992; Fukatsu and Nikoh, 1998). Once a pattern of relationship is established this method may be applied using

whole insect DNA. It should be noted that there are potential problems associated with PCR such as selective amplification of some sequences and hybrid formation, which are reviewed by Wintzingerode et al. (1997).

One additional approach to the detection of different kinds of organisms in the DNA samples is to use oligonucleotide primers that are complementary to the front of the 16S-rDNA and the middle of the 23S-rDNA. In bacteria in which the order of genes is 16S-23S (most species), there is a spacer region between the genes the length of which is labile and usually differs among species. If, after PCR, several bands are observed, they probably correspond to different bacteria. This approach has been applied to the cloning of P-endosymbionts of psyllids and to the putative S-endosymbionts which differ in the size of their 16S-23S spacer (Thao, M. L. and Baumann, P., unpublished observations).

Specific oligonucleotide primers also can be made complementary to unique sequences of the endosymbiont 16S and 23S rDNA. This has been done for *Buchnera* 16S rDNA (Rouhbakhsh et al., 1994). Another approach is to take advantage of unique linkage relationships. *Buchnera*-specific probes have been made which span the region *argS-16SrDNA* and *aroE-23SrDNA* (Munson et al., 1993; Rouhbakhsh and Baumann, 1995; Rouhbakhsh et al., 1994). The latter is especially useful, since most bacteria have 16S rDNA directly upstream of 23S rDNA.

Application

Aphids, psyllids, whiteflies, and mealybugs, which utilize plant sap as food, are of major economic importance in that they may cause plant debilitation and the transmission of a variety of plant pathogens (Gray and Banerjee, 1999; Sylvester, 1985). Tsetse flies, which suck blood, are important in the transmission of human and animal disease, especially in tropical regions of Africa (Harwood and James, 1979). Since these organisms are dependent on their P-endosymbionts for survival, an understanding of the genetics and physiology of the endosymbionts may be of use in devising strategies, which are based on interference with their endosymbionts, for the control of these insect pests. An additional approach, which is currently being developed, is the potential use of endosymbionts to produce compounds that would result in modification of the vector competence of an insect. Such an insect would be unable to harbor and/or transmit the disease agent. This approach has been discussed by Beard et al. (1993a) and Durvasula et al. (1997).

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