J Mol Evol (1995) 41:67–73

JOURNAL OF MOLECULAR EVOLUTION

Discovery and Molecular Characterization of a Plasmid Localized in *Buchnera* sp. Bacterial Endosymbiont of the Aphid *Rhopalosiphum padi*

Alma M. Bracho, David Martínez-Torres, Andrés Moya, Amparo Latorre

Departament de Genètica, Facultat de Ciències Biològiques, Universitat de València, 46100 Burjassot, València, Spain

Received: 6 May 1994 / Accepted: 18 July 1994

Abstract. We have identified and completely sequenced a novel plasmid isolated from the aphid *Rhopalosiphum padi.* Evidence which suggests that the plasmid occurs localized within the bacterial endosymbionts is presented. The plasmid contains the four genes that constitute the entire leucine operon. This fact makes it really unique since most plasmids are dispensable and lack genes that encode essential anabolic functions. Four more phloem-feeding aphid species also seem to contain homologous plasmids.

Although further work is necessary, we hypothesize that this plasmid has appeared during the evolution of the symbiotic association between the aphid and the bacterial endosymbiont. The fact that this plasmid contains the entire leucine operon can be related to physiological evidence showing that the aphid host's diet of plant phloem is deficient in essential amino acids.

Key words: Aphid — Endosymbiosis — Leucine operon — *Buchnera* — Plasmid

Introduction

Aphids (Homoptera: Aphidiodea) are plant-sap feeding insects that are dependent on an intimate association with eubacterial endosymbionts for normal growth and fecundity (Buchner 1965; Douglas 1989, 1992a; Houk 1987; Ishikawa 1989). Sequence comparison of the 16S ribosomal DNA (rRNAs) of primary endosymbionts of several aphid species, including *Rhopalosiphum padi,* reveals their close relationship to members of the γ-3 subdivision of the class Proteobacteria and their monophyletic origin (Munson et al. 1991a; Unterman et al. 1989). Munson et al. (1991b) proposed that the aphid primary endosymbionts should be classified as a novel species, *Buchnera aphidicola*—thus creating a new bacterial genus. The sequence-based phylogeny of *Buchnera* is completely concordant with the morphology-based phylogeny of their hosts, suggesting a single original infection in a common ancestor (Moran et al. 1993; Moran and Baumann 1994). Secondary endosymbionts of distinct lineage have also been described in several phloem-feeding insects, including aphids, although they are less ubiquitous and less abundant than primary ones (Campbell and Purcell 1993; Douglas 1989; Munson et al. 1991a).

The bulk of the primary symbiont population is found within aphid hypertrophied specialized cells called mycetocytes, which are generally accepted as being polyploid. Mycetocytes aggregate to form mycetomes localized in the hemocel beneath the digestive tract. The bacteria are maternally transmitted, never having a free-living phase, and to date, any attempt to culture them has failed.

Buchnera has been considered to be the ultimate factor responsible for the paradoxically high increase rates of aphids feeding on phloem sap, which represents a nutritionally poor diet with an exceptionally high carbon/nitrogen ratio, unbalanced amino acid composition, and low levels of lipids and vitamins (Dixon 1975; Houk and Griffiths, 1980; Raven 1983; Douglas 1992b; Douglas and Prosser 1992; Sasaki et al. 1991). The most plausible

Correspondence to: A. Latorre, Departament de Genètica, Facultat de Ciències Biològiques, Universitat de València, c/ Dr. Moliner, 50., 46100 Burjassot, València, Spain

hypothesis for the role of the symbionts is in "nitrogen upgrading," i.e., their participation in the synthesis of essential amino acids from nonessential ones, although the precise mechanisms are not yet established (Prosser et al. 1992).

In the present paper we report the discovery and molecular characterization of a multicopy plasmid from *Buchnera* housed in the aphid *Rhopalosiphum padi,* which codes for the entire operon of an essential amino acid: leucine. Why such an operon has escaped from the bacterial chromosome and become an autonomous replicating entity is not clear but some ideas are put forward on this matter.

Materials and Methods

DNA Isolation, Digestion, and Hybridization. The DNA isolation method is a protocol that has been used to isolate mitochondrial DNA (mtDNA) from aphids which uses a modified alkaline lysis protocol that preferentially extracts small, closed, covalently circular molecules such as mtDNA in insects (Latorre et al. 1986; Martínez et al. 1992). The DNA so extracted was digested with the following restriction endonucleases: *Eco*RI, *Hae*III, *Hin*dIII, *Xba*I, and *Pst*I. Restriction fragments were directly visible on agarose gels after staining with ethidium bromide (0.1 μg/ml) by exposing the gels on an ultraviolet transilluminator. Extra non-mtDNA fragments were always visualized on the gels. Probes for hybridizations were nonradioactively labeled with digoxigenin-11-dUTP (Martínez et al. 1992) and applied to Southern blots. Labeling, hybridization, washing, and detection of the hybrids were carried out basically following the supplier's instructions (Boehringer Mannheim).

Cloning and Sequencing Analysis. A 7.4-kb fragment and two 3.5-kb fragments obtained from *Eco*RI and *Pst*I digestion, respectively, were cloned in plasmid pUC18 following standard procedures (Ausubel et al. 1987). The recombinant plasmids were named p7.4E, p3.49P, and p3.53P, respectively. Sequences were obtained, according to the method of Sanger, from serial nested deletions (Henikoff 1984) of p3.49P and p3.53P and also from partial direct sequencing from both ends of all *Xba*I, *Hin*dIII, and *Pst*I subclones of the p7.4E derivative. The sequence submitted to the EMBL database was assigned the accession number number X71612. Analysis of the nucleotide sequence was carried out with version 7 of the GCG software package. Comparisons with the GenBank database and gene identification were made using the TFastA program. Alignments, searches for putative Ter sequences, G + C content analysis, identification of open reading frames, and searches for transcription terminators were achieved using Gap, Findpatterns, Composition, Frames, and Terminator programs, respectively.

Nuclease S1 and Deoxyribonuclease I (DNAseI) Assays. DNA extracted from whole aphids as described earlier was separately digested with nuclease S1 and DNAseI following standard procedures. Nuclease S1 (Pharmacia) digestions were carried out at 37°C for 30 min with 5 units of the enzyme in a volume of 100 μl containing about 100 ng of DNA. To stop reactions, 1 μl 0.5 M EDTA was added and afterward DNA was ammonium-isopropanol precipitated and resuspended in an appropiate volume of 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. DNAseI (Boehringer Mannheim) digestions were performed in a 25-μl reaction volume containing about 100 ng DNA basically following the procedure described for nick-translation (Ausubel et al. 1987). A $1/10^5$ dilution of the enzyme stock (1 mg/ml) was used. Five incubation times at 21°C were allowed ranging from 30 s to 30 min. To stop reactions 1 μl 0.5 M EDTA was added and afterward DNA was precipitated as described for the S1 assay.

In Situ *Localization on Paraffin Sections.* Fixation of the aphids, embedding, and sectioning were performed as described (Ausubel et al. 1987). Xylene-deparaffined and dehydrated slides were treated with lysozyme (25 mg/ml in PBS, 2 min) and afterward with proteinase K (100 μg/ml in PBS, 15 min, 37°C). Glycine 0.2% in PBS was added to stop proteinase digestion. Slides were then postfixed (30 min), rinsed in PBS and 2× SSC, and dehydrated. Chloroform was added for 15 min and after two 15-min washings in 100% and 95% ethanol, slides were allowed to air dry before proceeding to the hybridization steps. Prehybridization was performed for 30 min at 42°C in 50 μl 6× SSC, 45% formamide, 5× Denhardt's solution, and 100 μg/ml denatured herring sperm DNA. Hybridization, washings, and detection of the hybrids were carried out following an experimental procedure originally developed for hybridization of *Drosophila* polytene chromosomes with digoxigenin-dUTP-labeled probes (de Frutos et al. 1989).

Bacterial Isolation and Plasmid Isolation. Isolation of *Buchnera* was carried out following the method described by Whitehead and Douglas (1993). Plasmid DNA was also identified using the in-well lysis and electrophoresis procedure of Eckhardt (1978). The pellets from endosymbiont isolation and from bacterial culture for molecular size markers were quickly and carefully resuspended in a 20-μl lysis mixture containing 20% Ficoll 400, 0.05% bromophenol blue, 10 μl/ml of RNAse A, and 1 mg/ml of lysozyme in Tris-borate-EDTA buffer. The suspension was immediately transferred into the well of a vertical 0.8% agarose gel. Then 30 μl of a sodium dodecyl sulfate (SDS) mixture (0.2% SDS, 10% Ficoll 70 in TBE) was carefully layered on top of the bacterial sample and 100 μl of an overlay mixture (0.2% SDS, 5% Ficoll 70 in TBE) was added without disturbing the SDS-lysozyme layers. The slots were sealed with molten agarose, and the plasmid DNA was separated by electrophoresis at 4 mA for 30 min, at 33 mA for 15 min, and at 15 mA for 3 h. The DNA was then transferred to a membrane and hybridized with the p7.4E probe. Two overnight cultures of *E. coli* transformed with recombinant plasmid p7.4E and p2.2X (a derivative from p7.4E) were used as size markers. Total sizes of p7.4E and p2.2X are 10.1 kb and 4.9 kb, respectively.

Results

Detection and Preliminary Characterization of a New DNA Molecule

MtDNA extractions of *Rhopalosiphum padi* always yielded extra nonmitochondrial DNA fragments in restriction digests since these fragments failed to hybridize with mtDNA probes (Martínez et al. 1992). Different patterns of extra bands were obtained with different restriction enzymes but restriction mapping revealed that all these bands belonged to the single fragment of about 7.8 kb yielded in *Hae*III digestions. Their relative intensities on gels were comparable to that of mtDNA. To allow further characterization, major fragments obtained in digestions with *Eco*RI (7.4 kb) and *Pst*I (two different fragments of about 3.5 kb each) were cloned (Fig. 1). The recombinant plasmids—named, respectively, p7.4E (*Eco*RI fragment), p3.49P and p3.53P (*Pst*I fragments)—all together contained the entire molecule as verified by restriction mapping.

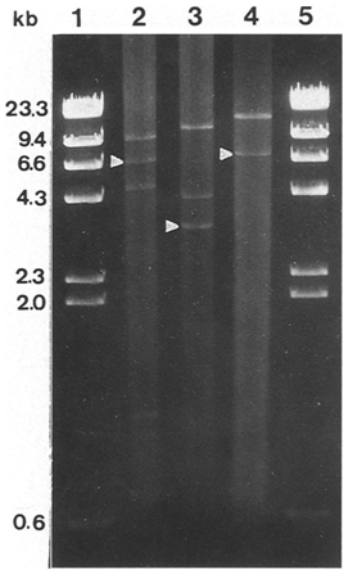

Fig. 1. mtDNA extracted from *R. padi* and digested with several restriction enzymes. Lanes 2, 3, and 4 correspond to *Eco*RI, *Pst*I, and *Hae*III digestions, respectively. *Arrowheads* indicate those fragments that failed to hybridize against mitochondrial DNA probes. Other visible bands correspond to mitochondrial DNA. Lanes *1* and *5* are λ DNA digested with *Hin*dIII as size standard. Sizes in kb are indicated on the left.

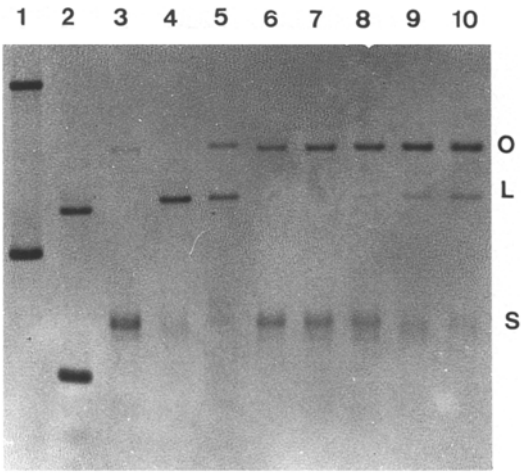

Fig. 2. Nuclease S1 and DNAseI assays from whole aphid mtDNA extraction. Lanes *1* and *2* are undigested recombinant plasmids p7.4E and p3.53P, having a size of about 10 kb and 6 kb, respectively, used as molecular size markers for supercoiled DNA (*bottom band*) and open circular DNA (*upper band*). Lane *3* corresponds to undigested DNA. Lane *4* is a *Hae*III digestion. Lane *5* is a nuclease S1 digestion. Lanes *6–10* are DNAaseI digestions at 30 s, 1 min, 5 min, 15 min, and 30 min, respectively. The gel was transferred and hybridized against the p7.4E probe.

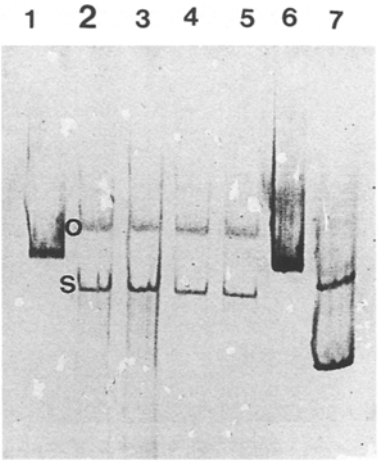

Fig. 3. Hybridization with p7.4E probe after Eckhardt procedure on isolated bacteria. Lanes *1, 6,* and *7* are molecular markers that correspond to 10.-kb supercoiled DNA (lanes *1* and *6*) and to 4.9-kb supercoiled and open circular DNA (lane *7*). Lanes 2–*5* correspond to supercoiled (*s*) and open circular (*o*) forms of plasmid pRPE.

The restriction map obtained was circular, although it was not clear that this molecule was so, since repetitive DNA would also yield a circular map. Since the method used in mtDNA extraction (Latorre et al. 1986; Martínez et al. 1992) was based on an alkaline method developed to isolate small, covalently closed, circular DNA, the circular nature of this molecule seemed plausible. To confirm that this molecule was circular and that it was not repetitive DNA, nuclease S1 and DNAseI assays were performed (Fig. 2). After hybridization with p7.4E probe two bands were detected in undigested DNA that corresponded to supercoiled and open circular DNA. Nuclease S1 digestion linearized most supercoiled DNA, yielding a band of the same size as that obtained in *Hae*III digestion, which linearizes this molecule. As expected, open circular DNA was unaffected by nuclease S1 treatment. DNAseI digestions demonstrated that the longer the incubation time, the less the amount of supercoiled DNA and the more the amount of open circular DNA, also appearing at longer times bands corresponding to linearized DNA as a result of DNAaseI nicking in both DNA strands. The Eckhardt method applied to isolated bacterial endosymbionts yielded two bands whose migration in the gel corresponded to the supercoiled and open circular forms obtained respectively from whole applied DNA extractions (Fig. 3). These results preclude a repetitive chromosomal nature for this molecule and confirm its circularity. Along with the in vivo localization carried out against aphid sections (see below), the fact that this circular molecule can be obtained intact from isolated bacteria demonstrates its bacterial origin.

Gene Content

The deduced gene content of the completely sequenced circular molecule along with the restriction map are shown in Fig. 4. Four of the six genes identified belong to the *leu* operon. They are 70.5%, 76.6%, 75.2%, and 66.0% similar respectively to genes *leuA, leuB, leuC,* and *leuD* from *Salmonella typhimurium* and 81.4%, 76.6%, 80.4%, and 63.5% respectively to the corresponding genes in *Escherichia coli.* The other two genes, 71% similar to each other, failed to yield a clear homology with any sequences in the database. The closest similar-

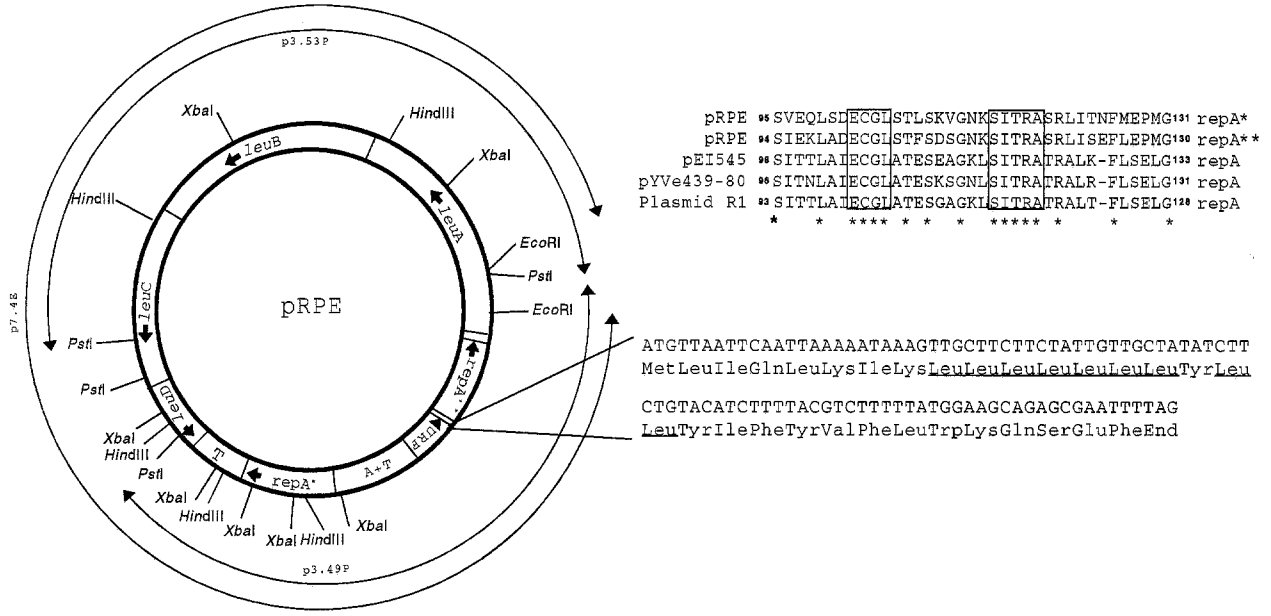

Fig. 4. Gene content and putative functional regions of the *R. padi* endosymbiont plasmid pRPE. The three originally cloned fragments are indicated. Extracted from the figure appears the sequence of the leader peptide with the nine almost consecutive leucines *underlined.* The alignment of part of the amino acid sequence including the central highly conserved motifs of both pRPE *repA* genes with the corresponding sequences from several enterobacterial plasmids is shown on the upper right side. Identities in all five sequences are indicated by *asterisks* and the two highly conserved central motifs are *boxed.* Directions of transcription for all the genes are indicated by *arrowheads.* The A + T-rich region is indicated. *T* indicates the location of a sequence showing similarity with the *Ter* consensus sequence from the *E. coli* chromosome and other related replicons.

ities were with plasmid *repA* genes, which code for plasmid-specific replication initiation protein RepA, from different enterobacterial species (e.g., pE1545 from *Klebsiella pneumoniae,* GenBank M93064; pYVe439–80 from *Yersinia enterocolitica,* GenBank M55182; plasmid R1 from *Escherichia coli,* GenBank V00351 and other R1 related plasmids). All these *rep*A genes when aligned with these two putative *repA* genes reveal two highly conserved short amino acid motifs of unknown function in the central region of the peptide (Fig. 4). When this motif was used to search homologous sequences in the database, only plasmid *repA* genes matched the consensus. This finding supports the idea that these genes are in fact two *repA* genes belonging to the same circular molecule as the *leu* operon genes. One of these two *repA* genes is transcribed in the opposite direction with respect to all the other genes. Close upstream to the *repA* gene that precedes the four *leu* genes there is a leader peptide of 31 amino acids, nine of which are almost consecutive leucines coded by rare codons (Clark et al. 1992a); 3′ downstream from this peptide several attenuator-forming sequences were found. Overlapping with the leader peptide, but in a different reading frame, is an open reading frame consisting of 166 amino acids. No similarities have been found between this peptide and any protein in the databases. In summary, all these data point toward the plasmid nature of this new molecule that will be named as pRPE (from plasmid of *Rhopalosiphum padi* endosymbiont).

Although the precise replication mechanism of pRPE cannot be directly deduced from sequence data, in addition to *rep*A genes, other features associated with replication processes have been identified: an extremely A + T-rich region (87%) that could contain the origin of replication (Kowalski et al. 1988) and a Tus binding site (*Ter* sequence) involved in replication arrest (Hill 1992). No *DnaA* boxes were identified in agreement with what has been described for the *Buchnera* (Lai and Baumann 1992). It is also worth noting that the high A + T content of this plasmid (74%) has been also reported for other *Buchnera* genes. (For a review see Moran and Baumann 1994.)

In Vivo Localization

Figure 5a shows a cross section of the abdomen of an adult aphid which was hybridized with the p7.4E probe. At least five mycetocytes embryos cross-sectioned in the ovarioles as well as maternal mycetocytes were stained. In both cases it was evident that the p7.4E probe attaches to cytoplasm that housed *Buchnera* but the nuclei of the mycetocyte do not at all. Figure 5b shows an enlarged image of a cross-sectioned embryo from Figure 5a. Therefore the in situ hybridization on aphid sections confirmed the presence of pRPE restricted to both maternal and embryonic mycetocytes evidencing its presence in the primary symbionts housed by *R. padi.* Since the Eckhardt method indicated that only supercoiled and open

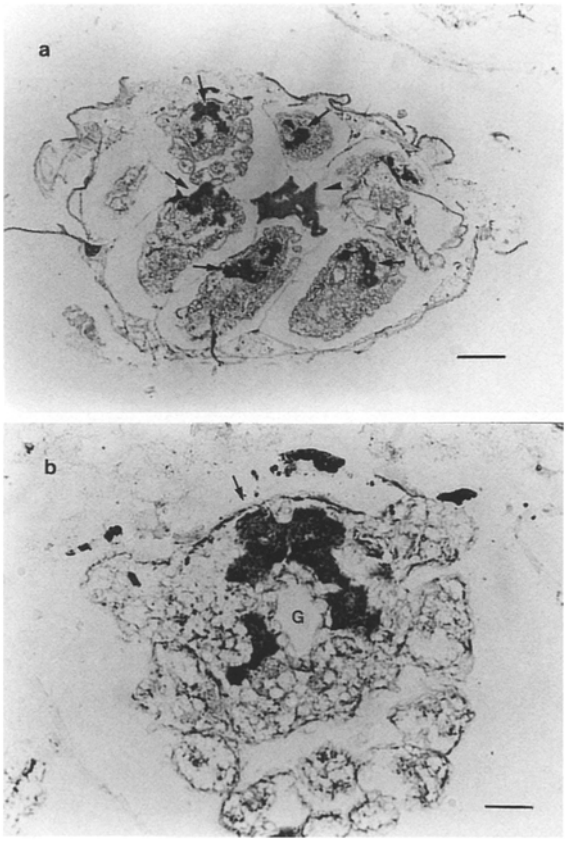

Fig. 5. Phase-contrast microscopic image of nonradioactive *in situ* hybridization of *R. padi* sections against the 7.4-kb *Eco*RI fragment of pRPE. **a** A 5-μm cross section of the abdominal part of an adult aphid. *Arrows* indicate mycetocytes corresponding to embryos while *arrowheads* point out the maternal mycetocytes. **b** An enlarged image of an embryo showing the mycetocyte. *G* indicates embryonic gut tube. *Bars* represent 100 μm in **a** and 25 μm in **b.**

circular plasmid DNA could be detected and there was no evidence of chromosomal hybridization (Fig. 3), it appears that the positive in situ hybridization (Fig. 5) was due to the presence of the plasmid in these bacteria.

Presence in Other Aphid Species

The hybridization of DNA extracted from additional aphid species revealed the presence of the same or a quite similar plasmid. Figure 6 shows the digests of circular DNA-enriched fractions from five species of gramineae feeding aphids (*Rhopalosiphum padi, Rhopalosiphum maidis, Schyzaphis graminum, Sitobion avenae,* and *Metopolophium festucae*). It should be noticed that in order to get comparable sizes for the corresponding non-mtDNA extra bands, different restriction enzymes were used for each species. It is evident that all these five species have endosymbionts carrying DNA molecules (most probably plasmids) that are homologous to pRPE, and also of similar size.

Discussion

Plasmids are known to confer many dispensable properties. At present, genes that code for essential anabolic functions have not yet been described in prokaryotic plasmids. This is the first time the complete sequence of a bacterial plasmid bearing genes of an operon encoding for the synthesis of an essential amino acid has been reported. The high similarities found between this operon and the leucine operon from *S. thyphimurium* and *E. coli* are not surprising since *Buchnera* has been assigned to the γ-3 subdivision of the class Proteobacteria in which enterobacteriaceae are included (Munson et al. 1991a,b). Furthermore, the two *repA* genes present in pRPE bear only a resemblance to *repA* genes from enterobacteriaceae plasmids belonging to the IncFII group. Apparently the leucine operon typically present as a single copy in the large bacterial replicon has escaped, becoming an autonomously replicating entity. However, our results do not preclude that a copy of the operon remains in the bacterial chromosome, since we do not know whether the hybridization method was sensitive enough to detect single-copy genes. The structure of this operon suggests that a transcription attenuation mechanism operates similar to that described for the *leu* operon of *S. typhimurium* (Gemmill et al. 1979). The 31-amino-acid leader peptide, nine of which are almost adjacent leucines (Fig. 3), and the fact that these leucines are encoded by codons that should be rare in *Buchnera* (Clark et al. 1992a) suggest that minimum levels of leucyl-tRNA^{leu} are more strictly controlled (Bartkus et al. 1991) than those described for *S. typhimurium* and *E. coli.* Moreover, plasmid copy-number is also probably regulated by leucyl-tRNA^{leu} levels since one of the two existing *repA* genes is intercalated between the leader peptide and the first *leu* gene, and synthesis of the RepA protein is rate-limiting for initiation of replication (Womble and Rownd 1988).

Why, more so than other essential amino acids, should leucine have strictly controlled, and probably enhanced, expression and independent regulation in the aphid symbiont? Exogenous leucine is a major effector of the leucine-lrp regulon which is known to control simultaneously many genes and operons promoting a global response mechanism in *E. coli* (D'Ari et al. 1993; Newman et al. 1992) to cope with nutritionally different situations. Its presence in the external medium would indicate a feast regime (e.g., intestinal habitat) activating degradation of compounds in the external medium and population growth and repressing biosynthetic functions, while its absence activates the biosynthetic pathways.

Aphid mycetocyte symbionts are probably the major source of most, perhaps all, of the insect's essential amino acids (Douglas and Prosser 1992), suggesting that symbiotic bacteria regulate their synthesis and transport differently than do their free-living relatives (Margulis 1991; Douglas 1992a). The precise mechanisms under-

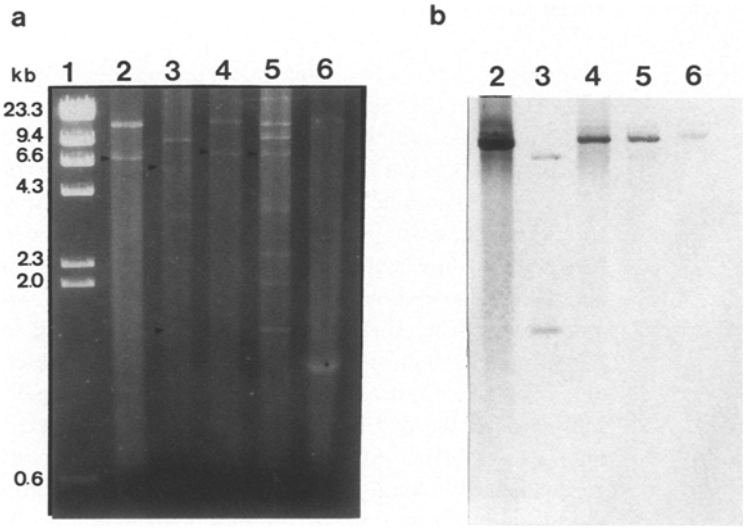

Fig. 6. **a** Digestions of circular DNA enriched fractions from five species of gramineae feeding aphids. Lane *1* is λ DNA digested with *Hin*dIII as size standard. Sizes in kb are indicated on the left. Lanes *2–6* correspond to restriction-enzyme digestions of DNA extracted from *Rhopalosiphum padi* (*Hae*III), *Rhopalosiphum maidis* (*Eco*RI), *Schyzaphis graminum* (*Pst*I), *Sitobion avenae* (*Eco*RV), and *Metopolophium festucae* (*Hin*dIII). **b** Hybridization of the same gel with the cloned 7.4-kb *Eco*RI fragment which comprises almost the whole pRPE plasmid. Probably due to the small amount of DNA obtained in lane *6* **(a),** *M. festucae* apparently lacks the plasmid but its presence is revealed after hybridization, as can be seen in lane *6* **(b).**

lying this regulation are still far from clear, but a leucine-mediated regulation through the lrp regulon such as that described for *E. coli* could be hypothesized. pRPE, by apparently regulating leucine synthesis so precisely, might play an essential role in such a regulatory mechanism.

One feature of pRPE that supports its integration in a symbiotic system is its high A + T content (74%). This reinforces the idea that in *Buchnera* the accumulation of A and T has taken place all over the whole genome (Ohtaka and Ishikawa 1993) in the process of adaptation to the mycetocyte environment. This tendency to increase the A + T content seems to be most characteristic of genomes of those species adapted to intracellular environments such as parasitic prokaryotes, prokaryotic endosymbionts of many insect species, and the mitochondria (at least in arthropoda) which represent a final state in the integration process of an endosymbiont (Ohtaka and Ishikawa 1993; Wolstenholme and Jeon 1992).

The ubiquity of pRPE (or other extremely related plasmids), in at least five aphid species that feed on nitrogen-depleted fluid from grasses, generalizes our finding (Fig. 6). Many other insects, probably all homopterans feeding on plant sap, carry ovum-transmitted cytoplasmic endosymbionts, e.g., leafhoppers (Campbell and Purcell 1993), whiteflies (Clark et al. 1992b), as well as coleopteran weevils (Campbell et al. 1992). Attempts to find similar plasmids in mycetocyte-bearing insects should reveal the genetic and nutritional basis for these ancient bacterial associations (Buchner 1965).

Acknowledgments. We thank F.J. Ayala and L. Birkle for the critical reading of the manuscript. This work has been supported by grants PB90-0429 and 92/CPE-055 from the "Dirección General de Investigación Científica y Técnica" and the "Institució Valenciana d'Estudis i Investigació," respectively, to A.M. and fellowships from the "Ministerio de Educación y Ciencia" to A.M.B. and D.M.T.

References

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds) (1987) Current protocols in molecular biology. Wiley, New York

Bartkus JM, Tyler B, Calvo JM (1991) Transcription attenuation-mediated control of *leu* operon expression: influence of the number of leu control codons. J Bacteriol 173:1634–1641

Buchner P (1965) Endosymbiosis of animals with plant microorganisms. Interscience Publishers Inc., New York

Campbell BC, Bragg TS, Turner CE (1992) Phylogeny of symbiotic bacteria of four weevil species (Coleoptera: Curculionidae) based on analysis of 16S ribosomal DNA. Insect Biochem Mol Biol 22: 415–421

Campbell BC, Purcell AH (1993) Phylogenetic affiliation of BEV, a bacterial parasite of the leafhopper *Euscelidius variegatus,* on the basis of 16S rDNA sequences. Curr Microbiol 26:37–41

Clark MA, Baumann L, Baumann P (1992a) Sequence analysis of an aphid endosymbiont DNA fragment containing rpoB (β-subunit of RNA polymerase) and portions of rplL and rpoC. Curr Microbiol 25:283–290

Clark MA, Baumann L, Munson MA, Baumann P, Campbell BC, Duffus JE, Osborne LS, Moran NA (1992b) The eubacterial endosymbionts of whiteflies (Homoptera: Aleyrodoidea) constitute a lineage distinct from endosymbionts of aphids and mealybugs. Curr Microbiol 25:119–123

D'Ari R, Lin RT, Newman EB (1993) The leucine-responsive regulatory protein: more than a regulator? Trends Biochem Sci 18:260–263

De Frutos R, Kimura K, Peterson KR (1989) *In situ* hybridization of *Drosophila* polytene chromosomes with digoxigenin-dUTP-labeled probes. Trends Genet 5:366

Dixon AFG (1975) Aphids and translocation. In: Zimmermann MH, Milburn JA (eds) Encyclopedia of plant physiology, vol 1, New Series. Springer-Verlag, Berlin, pp 154–170

Douglas AE (1989) Mycetocyte symbiosis in insects. Biol Rev 64:409–434

Douglas AE (1992a) Symbiosis in evolution. Oxf Surv Evol Biol 8:347–382

Douglas AE (1992b) Requirement of pea aphid (*Acyrthosiphon pisum*) for their symbiotic bacteria. Entomol Exp Appl 65:195–198

Douglas AE, Prosser WA (1992) Synthesis of the essential amino acid tryptophan in the pea aphid (*Acyrthosiphon pisum*) symbiosis. J Insect Physiol 38:565–568

Eckhardt T (1978) A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. Plasmid 1:584–588

Gemmill RM, Wessler SR, Keller EB, Calvo JM (1979) *leu* operon of *Salmonella typhimurium* is controlled by an attenuation mechanism. Proc Natl Acad Sci U S A 76:4941–4945

Henikoff S (1984) Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene 28:351–359

Hill TM (1992) Arrest of bacterial DNA replication. Ann Rev Microbiol 46:603–633

Houk EJ (1987) Symbionts. In: Minks AK, Harrewijn P (eds) Aphids, their biology, natural enemies and control, vol 2A. Elsevier Biochemical Press, Amsterdam, pp 123–129

Houk EJ, Griffiths GW (1980) Intracellular symbiotes of the Homoptera. Ann Rev Entomol 25:161–187

Ishikawa H (1989) Biochemical and molecular aspects of endosymbiosis in insects. Int Rev Cytol 116:1–45

Kowalski D, Natale DA, Eddy MJ (1988) Stable DNA unwinding, not breathing, accounts for single-strand-specific nuclease hypersensitivity of specific A + T-rich sequences. Proc Natl Acad Sci U S A 85:9464–9468

Lai C-Y, Baumann P (1992) Genetic analysis of an aphid endosymbiont DNA fragment homologous to the rnpA-rpmH-dnaA-dnaN-gyrB region of eubacteria. Gene 113:175–181

Latorre A, Moya A, Ayala JF (1986) Evolution of mitochondrial DNA in *Drosophila subobscura.* Proc Natl Acad Sci U S A 83:8649–8653

Margulis L (1991) Symbiogenesis and symbionticism. In: Margulis L, Fester R (eds) Symbiosis as a source of evolutionary innovation. MIT Press, Cambridge MA, pp 1–14

Martínez D, Moya A, Latorre A, Fereres A (1992) Mitochondrial DNA variation in *Rhopalosiphum padi* (Homoptera: Aphididae) populations from four spanish localities. Ann Entomol Soc Am 85:241–246

Moran NA, Munson MA, Baumann P, Ishikawa H (1993) A molecular clock in endosymbiotic bacteria is calibrated using the insect hosts. Proc R Soc Lon [B] 253:167–171

Moran NA, Baumann P (1994) Phylogenetics of cytoplasmically inherited microorganisms of arthropods. Trends Ecol Evol 9:15–20

Munson MA, Baumann P, Clark MA, Baumann L, Moran NA, Voegtlin DJ, Campbell BC (1991a) Evidence for the establishment of aphid-eubacterium endosymbiosis in an ancestor of four aphid families. J Bacteriol 173:6321–6324

Munson MA, Baumann P, Kinsey MG (1991b) *Buchnera* gen. nov. and *Buchnera aphidicola* sp. nov., a taxon consisting of the mycetocyte-associated, primary endosymbionts of aphids. Int J Syst Bacteriol 41:566–568

Newman EB, D'Ari R, Lin RT (1992) The leucine-Lrp regulon in *E. coli:* a global response in search of a raison d'être. Cell 68:617–619

Ohtaka C, Ishikawa H (1993) Accumulation of adenine and thymine in a groE homologous operon of an intracellular symbiont. J Mol Evol 36:121–126

Prosser WA, Simpson SJ, Douglas AE (1992) How an aphid (*Acyrthosiphon pisum*) symbiosis responds to variation in dietary nitrogen. J Insect Physiol 38:301–307

Raven JA (1983) Phytophages of xylem and phloem: a comparison of animal and plant sap-feeders. Adv Ecol Res 13:136–234

Sasaki T, Hayashi H, Ishikawa H (1991) Growth and reproduction of the symbiotic and aposymbiotic pea aphids, *Acyrtosiphon pisum,* maintained on artificial diets. J Insect Physiol 37:749–756

Untermann BM, Baumann P, McLean DL (1989) Pea aphid symbiont relationships established by analysis of 16S rRNAs. J Bacteriol 171:2970–2974

Whitehead LF, Douglas AE (1993) A metabolic study of *Buchnera,* the intracellular bacterial symbionts of the pea aphid *Acyrthosiphom pisum.* Gen Microbiol 139:821–826

Wolstenholme DR, Jeon KW (1992) Mitochondrial genomes. International review of cytology, vol 141. Academic Press, New York

Womble DD, Rownd RH (1988) Genetic and physical map of plasmid NR1: comparison with other IncFII antibiotic resistance plasmids. Microbiol Rev 52:433–451