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Intracellular Bacterial Symbionts of Aphids Possess Many Genomic Copies per Bacterium

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Abstract. Although *Buchnera*, the endosymbiotic bacteria of aphids, are close relatives of *Escherichia coli*, their genome size is only a seventh that of *E. coli*. In this study, we estimated the genomic copy number of *Buchnera* by dot-blot hybridization and fluorimetry using a video-intensified microscope photon-counting system and obtained convincing evidence that each cell of these bacteria contains an average of 120 genomic copies. Thus, the *Buchnera* symbiont, with many copies of a small-sized genome, is reminiscent of cell organelles such as mitochondria and chloroplasts.

Key words: *Buchnera* — Pea aphid — Symbiosis — Genomic copy number — Dot-blot analysis — Microscope photon-counting system — Polyploidy

Introduction

Many eukaryotic cells provide the sole habitat for a vast and varied array of prokaryotic lineages (Buchner 1965). These intracellular associations have evolved repeatedly and have had major consequences for the diversification of both prokaryotic symbiont and host. The magnitude of these consequences is immediately evident if one considers the examples of mitochondria and chloroplasts, now widely acknowledged to be descended from prokaryotes that invaded the intracellular habitat (Margulis 1970; Gray and Spencer 1996). A typical example of these associations is the intracellular symbiosis between aphids (Homoptera, Aphididae) and prokaryotic cells, *Buchnera* (Munson et al. 1991), which dwell in the cytoplasm of aphid somatic cells called bacteriocytes, or mycetocytes (Griffiths and Beck 1973). The host and *Buchnera* are intimately mutualistic and indispensable to each other for their growth and reproduction (Ishikawa 1989; Baumann et al. 1995; Douglas 1998). While *Buchnera* belong to the γ3 group of the Proteobacteria and have *E. coli* and related bacteria as their closest relatives (Unterman et al. 1989), they can no longer grow outside the bacteriocyte (Ishikawa 1984).

Phylogenetic analysis based on 16S rDNA sequences of *Buchnera* from a number of aphid species, together with paleontological and morphological studies on the lineage of these insects, argues for a monophyletic origin of *Buchnera* and suggests the following evolutionary scenario (Moran et al. 1993; Moran and Baumann 1994): about 200–250 million years ago a common ancestor of present-day aphids was infected with a close relative of *E. coli* and an intracellular association was established. Subsequent transmission of the symbiont through each host generation led to parallel divergence and cospeciation of the symbiont and host, resulting in the present species of aphids and those of *Buchnera* (Moran and Baumann 1994). Our main interest is to know what has happened to the bacteria that have been confined in eukaryotic cells for such a long period. Our recent data indicate that symbiosis has induced a dramatic reduction in the genome size of the bacteria (Charles and Ishikawa 1998). On the other hand, according to our observations, *Buchnera* cells are much larger in volume than *E. coli* cells (see Fig. 4a) and do not divide as frequently as

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free-living bacteria. These findings led us to suspect that, unlike the great majority of bacteria, *Buchnera* may be a bacterium with many copies of the genome in a single cell. Herein, we investigate the genomic copy number of *Buchnera* by two methods and report the first discovery of a bacterium with more than 100 genomic copies.

Materials and Methods

Insects, Bacteria, and Yeasts. A long-established parthenogenetic clone of pea aphids, *Acyrthosiphon pisum* (Harris), was maintained on young broad bean seedlings at 15°C with a 16-h photoperiod (Ishikawa 1982). Young apterous aphids were dissected in buffer A (35 m*M* Tris–HCl, pH 7.5, 25 m*M* KCl, 10 m*M* $MgCl_2$, 250 m*M* sucrose) under a microscope. Bacteriocytes were isolated from the insect body and collected by manual suction with a thin glass capillary. *Buchnera* cells were freed by pipetting bacteriocytes in buffer A and passing the suspension through an isopore membrane with a pore size of 3 μm to remove the nuclei of the bacteriocytes (Sasaki and Ishikawa 1995). *Buchnera* cells were washed in buffer A and collected by centrifugation at 1500*g*. The cells were suspended in buffer A and counted using a Thoma hemocytometer. After these procedures, virtually no contaminant particles were observed, and 2 to 4×10^7 *Buchnera* cells were recovered from 50 aphids. *E. coli* (DH5α) cells, grown in LB, were collected at the stationary phase, suspended in buffer A, and counted for their number as above. *Saccharomyces cerevisiae* (YPH497) cells, whose genome size is 13.56 Mbp, were used as a standard for calculating the DNA content by fluorimetry.

Isolation of Genomic DNA from Buchnera *and* E. coli *Cells.* By examining an aliquot under a microscope, the total cell number was estimated. Cells were treated with lysozyme and proteinase K, and their DNA was extracted according to the standard procedures of the phenol/chloroform/isoamyl alcohol method. Subsequently, they were treated with RNase A, followed by phenol/chloroform/isoamyl alcohol (Murray and Thompson 1980).

PCR Amplification of DNA Fragments. The *groE* operon of *E. coli* contains the *groES* gene, a 44-bp intergenic sequence, and the *groEL* gene, in this order from upstream (Tilly et al. 1981). Similarly, the *groE* operon of *Buchnera* consists of *groES* and *groEL* genes with a 47-bp intergenic spacer between them (Ohtaka et al. 1992). We amplified a 580-bp fragment of *E. coli groE* spanning its intergenic sequence, using the upstream primer A1 (5′-ATATTCGTCCATTGCATGATCG-3′, located in *groES*), the downstream primer B1 (5′-CGTTTGCTTTAGAGGCAACTTC-3′, located in *groEL*), and optimized PCR conditions as described. A 580-bp *groE* fragment of *Buchnera* was similarly amplified using the same primers and aphid total DNA as template. A 786-bp fragment of the internal region of *E. coli* 16S rDNA was amplified using the upstream primer C1 (5′-TGAGACACGGTCCAGACTCCTAC-3′) and the downstream primer D1 (5′-GTTGCGCTCGTTGCGGACTTAAC-3′). The amplified DNA fragments were labeled using [α-^{32}P]dCTP and the BcaBEST Labelling Kit (Takara).

Dot-Blot Hybridization (Kafatos et al. 1979). PCR-amplified DNA fragments and genomic DNA from *Buchnera* and *E. coli* were denatured by treatment at 95°C for 5 min in buffer B (10 m*M* Tris–HCl, pH 8.0, 150 m*M* NaCl, 10 m*M* EDTA) and quantitatively blotted onto Hybond N^+ membranes (Amersham Life Science) in a dilution series. DNA was fixed to the membrane by UV irradiation. After treatment in a solution containing herring sperm DNA, the DNA-bound membrane was incubated with a ^{32}P-labeled probe overnight at 42°C. The membrane was washed under stringent conditions, and the radioactivity remaining was quantified using a BAS-2500 image analyzer (Fujifilm).

Estimation of Cellular DNA Content by Fluorimetry. Staining was performed according to the method described by Suzuki et al. (1986). Cells were fixed in 75% ethanol and 25% acetic acid on ice for 60 min and washed in a dilution series of ethanol and water. The fixed cells were then treated with 0.1 mg/ml RNase A and stained with 2 μg/ml propidium iodide (PI). These stained cells were irradiated with UV light and examined under an epifluorescence microscope (BHS-RFK; Olympus) in the presence of an antifade reagent (SlowFade Antifade Kit Component A; Molecular Probes). Fluorescence intensities from individual cells were quantified using a video-intensified microscope photon-counting system [VIMPCS; Hamamatsu Photonics Ltd. (Kuroiwa et al. 1986)].

Results

Dot-Blot Analysis of the groE *Operon of* E. coli. The *E. coli* genome contains a single copy of *groE* (Blattner et al. 1997), an operon that codes for two molecular chaperones (Zeilstra-Ryalls et al. 1991). We amplified by PCR a 580-bp fragment of *groE* which contained parts of the two coding regions and the entire sequence of the intergenic spacer between them and blotted it quantitatively onto a membrane. We extracted genomic DNA from the same number of *E. coli* cells as the copy number of the *groE* fragments blotted above and blotted it onto the same membrane. The 580-bp *groE* fragment was labeled with ^{32}P and hybridized to the two membrane-bound DNA samples. As a result, approximately the same amount of radioactivity was detected from the two samples, reflecting the occurrence of one *groE* operon per *E. coli* cell (Fig. 1).

Dot-Blot Analysis of the groE *Operon of* Buchnera. The *Buchnera* genome also contains a single copy of *groE* (Charles and Ishikawa 1998). We performed a similar experiment using a PCR-amplified 580-bp fragment of *Buchnera groE* and the *Buchnera* genomic DNA. The 580-bp fragment was homologous to the 580-bp *E. coli* fragment above, with a sequence identity of 74% (Ohtaka et al. 1992). The *Buchnera groE* fragments and *Buchnera* genomic DNA were quantitatively blotted on a membrane and hybridized to the ^{32}P-labeled *Buchnera groE* fragments. Unlike the result in Fig. 1, the radioactivity detected from the genomic DNA was about 100 times greater than that detected from the *groE* fragment (Fig. 2). Since the genomic DNA was extracted from the same number of *Buchnera* cells as that of the *groE* fragment used, this result indicates that each *Buchnera* cell contains about 100 copies of the *groE* operon. Since the *groE* operon has been mapped to a single site on the entire genome of *Buchnera* (Charles and Ishikawa 1998), this result may suggest that the *groE* region is locally amplified and exists as numerous extrachromosomal elements. This is possible in view of the fact that *Buchnera*, when housed in the bacteriocyte, selectively

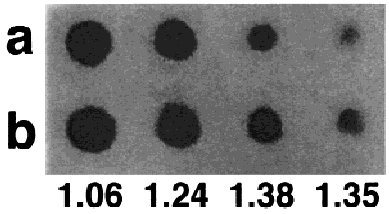

Fig. 1. Estimation of the copy number of the *groE* operon per *E. coli* cell. The PCR-amplified 580-bp *groE* fragments were purified by agarose gel electrophoresis, quantified by determination of UV absorption, and denatured as described under Materials and Methods. A 50% dilution series of this DNA sample was blotted on a Hybond N^+ membrane as *dots* from left to right in the upper row **(a)**, the leftmost dot containing 4×10^7 copies of the fragment. Genomic DNA was extracted from *E. coli* cells whose number had been estimated and denatured. A 50% dilution series was blotted from left to right in the lower row **(b)**. The concentration of the genomic DNA sample was adjusted so that the leftmost dot contained DNA from 4×10^7 cells. Thus, in each pair the upper and lower dots should have contained the same number of *groE* sequence, provided that the *groE* operon occurs only once in the *E. coli* genome. Blotted samples were fixed to the membrane by UV irradiation and incubated with a sufficient amount of the ^{32}P-labeled, denatured *groE* fragment overnight at 42°C. After extensive washing of the membrane, the radioactivity remaining with each dot was quantified using a BAS-2500 image analyzer. Shown below each pair of dots is the ratio of radioactivity detected from the genomic DNA (b) to that detected from the *groE* fragment (a).

produce a large amount of GroEL (Ishikawa 1982b; Ishikawa 1989), one of the *groE* products (Hara et al. 1990). Alternatively, the entire genome in the *Buchnera* cell may have been extraordinarily amplified.

Comparison of Copy Numbers of 16S rDNA Between E. coli *and* Buchnera. To test the two alternatives mentioned above, we compared copy numbers of the 16S ribosomal RNA gene (16S rDNA) between *E. coli* and *Buchnera.* For this purpose, an internal 786-bp fragment of *E. coli* 16S rDNA was amplified by PCR, labeled with ^{32}P, and used as a common probe. Genomic DNA was extracted from the same number of *Buchnera* and *E. coli* cells, blotted separately onto a membrane as dots in dilution series, and hybridized with sufficient amounts of the labeled rDNA fragment of *E. coli.* As a result, the radioactivity detected from *Buchnera* rDNA was about eight times more than that from *E. coli* rDNA (Fig. 3). In view of the sequence identity between the probe used and the corresponding region of *Buchnera* 16S rDNA (91.4%) (Munson et al. 1993), the real difference in copy number between *Buchnera* and *E. coli* rDNA could be more. It is well known that the *E. coli* genome contains seven copies of rDNA (Blattner et al. 1997). The *Buchnera* rDNA is different from that of *E. coli* in that the 16S and 23S rDNA genes are not arranged in a single operon (Munson et al. 1993) and that 16S and 23S rDNA are single-copy genes (Charles and Ishikawa 1998). Taken together, these results indicate that a *Buchnera* cell contains, at least, 60 copies of 16S rDNA.

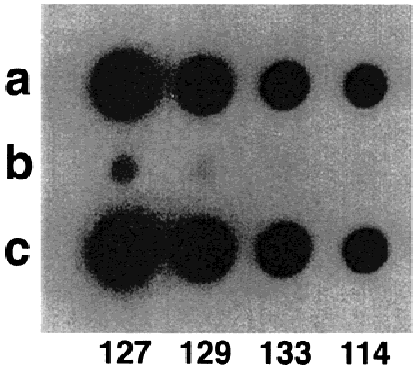

Fig. 2. Estimation of the copy number of the *groE* operon per *Buchnera* cell. A dot-blot hybridization analysis as in Fig. 1 was performed using PCR-amplified 580-bp *Buchnera groE* fragments, genomic DNA of *Buchnera,* and the ^{32}P-labeled *Buchnera groE* fragment as a probe. In the top row **(a)**, a 50% dilution series of the *Buchnera groE* fragments was blotted from left to right, the leftmost dot containing 4×10^8 copies of the fragment. The dots blotted in the second row **(b)** also contained *Buchnera groE* fragments, but the amount contained in each dot was 1% of the above, starting with 4×10^6 copies. Blotted in the third row **(c)** was a 50% dilution series of genomic DNA of *Buchnera,* starting with the DNA amount corresponding to 4×10^6 cells on the left. Thus, if the *groE* operon occurs once in the *Buchnera* genome, the dots in the second and third rows should have contained an equal number of *groE* sequences. Likewise, the dot in the top row should have contained 100 times as many *sym* sequences as that on the third row. Shown below the third row is the ratio of radioactivity detected from the genomic DNA (third row) to that detected from the *groE* fragment (top row), followed by multiplication by 100, since the radioactivity detected from dots in the second row was too weak for an accurate calculation.

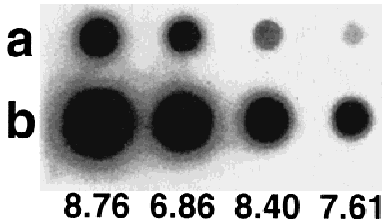

Fig. 3. Copy numbers of 16S rDNA per *E. coli* and *Buchnera* cell. Genomic DNA was extracted from *E. coli* and *Buchnera* cells, and a 50% dilution series was blotted from left to right, starting with the DNA amount corresponding to 4×10^6 cells for each bacterium. The membrane-bound DNA was hybridized with ^{32}P-labeled, 786-bp *E. coli* 16S rDNA fragments. Shown below each pair of dots is the ratio of radioactivity detected from *Buchnera* DNA **(b)** to that from *E. coli* DNA **(a)**.

Comparison of DNA Contents Among S. cerevisiae, E. coli, *and* Buchnera *Cells.* It would be an incredible coincidence that the two separate genes, *groE* and 16S rDNA, were locally amplified to the same extreme degree in a bacterial cell, suggesting that in *Buchnera* cells the whole genome itself has been amplified. To confirm this, we compared the quantity of genomic DNA extracted from the same number of *Buchnera* and *E. coli* cells by UV absorption. The result demonstrated that a *Buchnera* cell contains, at least, 10 times more DNA than an *E. coli* cell (data not shown). If one considers the

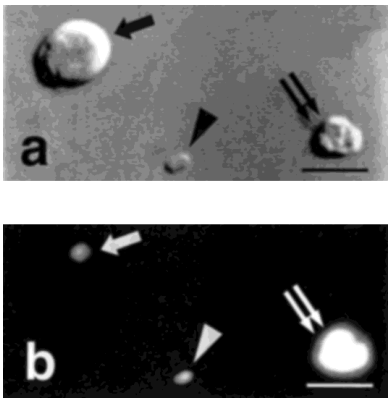

Fig. 4. Fluorescent photomicrograph of *S. cerevisiae*, *E. coli*, and *Buchnera* cells. Cells were fixed and stained with PI as described under Materials and Methods, and examined under an epifluorescence microscope. **a** Photomicrograph taken without UV irradiation. The *arrow*, *arrowhead*, and *double-arrow* indicate a haploid yeast, an *E. coli*, and a *Buchnera* cell, respectively. The bar represents 5 μm. **b** The same visual field as taken with UV irradiation. The *arrow*, *arrowhead*, and *double-arrow* indicate a haploid yeast nucleus, an *E. coli* cell, and a *Buchnera* cell, respectively.

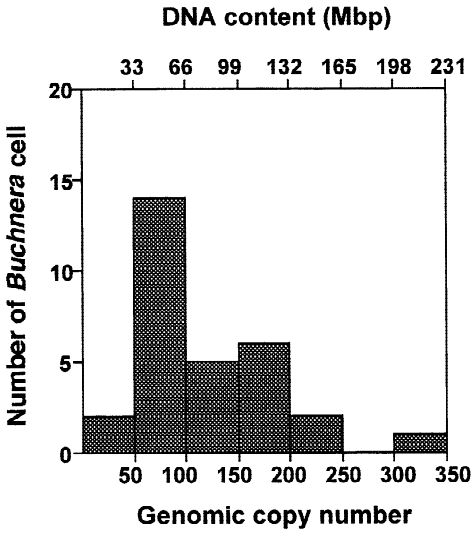

Fig. 5. Variation of DNA content and genomic copy number among 30 *Buchnera* cells. *Buchnera* cells were fixed, stained with PI, and irradiated with UV light as described under Materials and Methods. The fluorescence intensity emitted by each *Buchnera* cell was compared with that emitted by a haploid or diploid yeast cell in the same visual field using VIMPCS (Kuroiwa et al. 1986), and its DNA content was estimated on the basis of that of the yeast cell (13.56 or 27.1 Mbp). Linearity of fluorescence intensity emitted by a cell with its DNA content was confirmed by comparing among *E. coli* and haploid and diploid yeast cells in the same visual fields. Genomic copy number was estimated on the basis of the calculated DNA content and the genome size [0.657 Mbp (Charles and Ishikawa 1998)].

difference in the genome size of the two organisms (Charles and Ishikawa 1998; Blattner et al. 1997), this corresponds to a greater than 70-fold difference in the copy number of the genome in a single cell of *Buchnera* and *E. coli*. As a final step to look into the copy number of the *Buchnera* genome, we directly estimated the DNA content of individual cells of *Buchnera*, *E. coli*, and *S. cerevisiae*, by fluorimetry using VIMPCS (Kuroiwa et al. 1986) after staining with PI (Suzuki et al. 1986). We compared the fluorescence intensities emitted by these cells in the same visual fields and estimated the DNA content of each *Buchnera* cell by comparison with that of *S. cerevisiae*, whose DNA content per cell was known (Fig. 4). The result demonstrated that every *Buchnera* cell contains more DNA than the diploid yeast cell. In addition, it was revealed that *Buchnera* cells contain a variable amount of DNA. The fluorescence intensities emitted by yeast and *E. coli* cells in a single visual field were, as expected, nearly the same, but those emitted by *Buchnera* were variable from cell to cell, suggesting that this variation is not due to the techniques employed but due to intrinsic variation in the DNA content of individual *Buchnera* cells. The DNA quantity per *Buchnera* cell, calculated based on those of the haploid and diploid yeast cell, ranged from 30 to 130 Mbp in DNA length, and similar results were obtained when compared with *E. coli*. If one considers the genome size of *Buchnera* (Charles and Ishikawa 1998), this corresponds to a variation of 50 to 200 with respect to the genomic copy number per cell (Fig. 5). It was shown that a *Buchnera* cell, on average, contains 77-Mbp DNA, i.e., 120 copies of its genome. This result, taken together with those from the dot-blot analyses, presents convincing evidence that *Buchnera* are exceptional bacteria with many genomic copies.

Discussion

It has long been known that the bacteriocytes of aphids and other insects are polyploid. For the bacteriocyte of a cockroach, polyploidy as high as 512*n* has been reported (Buchner 1965). Our present study suggests that the bacterial symbiont harbored by the bacteriocyte also contains many genomic copies. The number of bacteriocytes in aphids tends to decline between embryo and adulthood (Wilkinson and Douglas 1998), but the size of individual bacteriocytes increases through larval development (Douglas and Dixon 1987). This suggests that the bacteriocyte is somehow prevented from dividing and increases its size in parallel with repeated replications of its genome. As for the *Buchnera* symbionts, they are also prevented from dividing by being packed in the bacteriocyte. It seems that *Buchnera* can increase in number only in accordance with an increase in the size of the host cell. It is probable that this spatial restriction intrinsically altered the bacteria so that the genome is repeatedly replicated without cell division. According to our earlier work, *Buchnera* cells isolated from the bacteriocyte syn-

thesize DNA vigorously but never divide (Ishikawa 1982a).

A dramatic reduction in the genome size (Charles and Ishikawa 1998) accompanied by an extreme increase in the genomic copy number in the intracellular bacterial symbiont is reminiscent of eukaryotic cell organelles such as mitochondria and chloroplasts (Gray and Spencer 1996). Loss of the ability to divide outside the eukaryotic cell is also a common attribute of *Buchnera* and these organelles. It is possible that these changes are an inevitable consequence for the genome of prokaryotes that have been housed in eukaryotic cytoplasm for an evolutionary length of time. The prolonged intracellular life may cause prokaryotes to abandon their common raison d'être of proliferating rapidly by frequent cell divisions. However, they still retain the ability to replicate their genome frequently, which may account for the extremely high number of the genomic copy of the symbiont and cell organelles. Both the slimming of the genome (Charles and Ishikawa 1998; Charles et al. 1997) and its A/T richness (Ishikawa 1987; Ohtaka and Ishikawa 1993) will tend to favor repeated replication.

From an evolutionary point of view, the high copy number of the *Buchnera* genome may explain how these symbiotic bacteria have slowed down Muller's (1964) ratchet, which otherwise would be serious to small and asexual populations such as that of *Buchnera* (Felsenstein 1977; Ohta 1987; Moran 1996). While in this study we investigated the genomic copy number of *Buchnera* cells harbored by bacteriocytes from adult insects, our unpublished results suggest that those harbored by embryonic bacteriocytes contain equally many genomic copies. It follows that *Buchnera* have retained their high genomic copy number for an evolutionary long time through each generation of the host insect. This raises the possibility that the multiple genomic copies in the *Buchnera* cell are not necessarily identical, but homologous, to each other. It may even be possible that there are multiple alleles on the genomes like those on homologous chromosomes of eukaryotes. Provided that recombination between these genomes takes place, it will bring together mildly deleterious mutations, from different loci of distinct genome copies, on a single genomic copy. Since the genomic copy harboring multiple deleterious mutations tends to be negatively selected, this process will effectively remove mildly deleterious mutations that otherwise accumulate in asexual populations. Thus, multiple genomes may enable *Buchnera* to utilize mechanisms somewhat similar to sexuality to avoid the deleterious effect caused by the bottleneck which occurs at each host generation when *Buchnera* are transmitted to progeny.

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