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Polyamine Composition and Expression of Genes Related to Polyamine Biosynthesis in an Aphid Endosymbiont, *Buchnera*

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Polyamine composition in an aphid endosymbiotic bacterium, *Buchnera* sp., was determined by high-performance liquid chromatographic analysis. We found that *Buchnera* contained virtually only a single polyamine, spermidine. The spermidine content of *Buchnera* was considerably higher in young aphids and tended to decrease with the age of the host. Expression of *speD* and *speE*, whose gene products are key enzymes in the synthesis of spermidine, was analyzed by real-time quantitative reverse transcription-PCR. It was shown that the levels of their mRNAs fluctuated in line with the spermidine content.

Buchnera spp. are intracellular symbiotic bacteria harbored by aphid bacteriocytes, cells specifically differentiated for this purpose (1, 3, 15). The symbiotic association between *Buchnera* and aphids is mutualistic and obligate in that neither partner can reproduce in the absence of the other (11). This is partly because *Buchnera* produce essential amino acids (6, 7, 20, 26) and vitamins (21), which are utilized by the host aphid. Molecular phylogenetic studies of 16S rRNA genes suggested that *Buchnera* belong to the γ subdivision of the *Proteobacteria* and that they are closely related to *Escherichia coli* (32). However, there are significant differences between *Buchnera* and *E. coli*. Each *Buchnera* cell has more than 100 copies of the genome (17), whose size is about a seventh of that of the *E. coli* genome (4). This suggests that these genomic copies must be stabilized in a specific way in the *Buchnera* cell. In the meantime, *Buchnera* cells do not divide as frequently as free-living bacteria, suggesting that their proliferation is strictly controlled by the host bacteriocyte (14). Since polyamines are known to be important factors for DNA stabilization, DNA replication, and cell proliferation, we directed our attention to these polycationic compounds.

Polyamines are linear aliphatic compounds that are positively charged under physiological ionic and pH conditions. They are present in all prokaryotic and eukaryotic cells and account for the majority of intracellular cationic charge (29). Among several functions implicated, charge neutralization of intracellular polyanions, especially DNA, may be the most important physiological role of polyamines. The interaction of polyamines with DNA induces such conformational changes as transitions from B to A and Z forms (30), bending (8), and, at higher polyamine concentrations, condensation of DNA (9, 24, 25). These polyamine-induced conformational changes may affect DNA metabolism and modify the interactions of DNA with sequence-specific DNA-binding proteins (23).

As the first step to investigating the roles of polyamines in *Buchnera*, in this study, we determined the polyamine composition of *Buchnera* and further assessed the expression of genes involved in the biosynthesis of polyamines.

MATERIALS AND METHODS

Host aphids. A long-established parthenogenetic clone of the pea aphid, *Acyrthosiphon pisum* Harris, was maintained on young broad bean plants, *Vicia faba* L., at 15°C in a long-day regimen of 16 h of light and 8 h of dark (13). Insects were collected within 24 h after larviposition by apterous mothers. These nymphs are described as 0-day aphids. Once the aphids reached adulthood, they were transferred twice a week to fresh plants in order to keep the nutritional conditions constant.

Isolation of *Buchnera*. The aphids were dissected in a drop of buffer A (35 mM Tris-HCl [pH 7.5], 25 mM KCl, 10 mM $MgCl_2$, 250 mM sucrose) (13) on a petri dish covered with 1% agarose gel. Bacteriocytes freed from the insect body were collected and gently crushed by pipetting. The homogenate was filtered through an isopore membrane filter (Millipore; pore size, 3 μm) to remove cell components of host origin. This filtration method was verified to give purer samples than other methods, such as the Percoll gradient method (26), and was applied to obtain DNA samples for *Buchnera* genome analysis (27). Shotgun sequencing of purified DNA detected no contaminant DNAs such as those of eukaryotic mitochondria or other bacteria (S. Shigenobu, personal communication), suggesting that this *Buchnera* sample was virtually free of contaminants.

Estimation of the volume of *Buchnera* cells used for HPLC analysis. An aliquot of isolated *Buchnera* cells was used to estimate the volume of *Buchnera* applied for high-pressure liquid chromatography (HPLC) analysis. The number of *Buchnera* cells was determined using hemocytometers. The volume of *Buchnera* cells, treated as spheres, was calculated from the diameter, measured with a micrometer. The sum volume of *Buchnera* cells was calculated by multiplying the number by the average volume.

***E. coli* strain.** *E. coli* TOP10 cells were cultured overnight at 37°C in LB medium and collected by centrifugation at the stationary phase.

HPLC analysis. *Buchnera* and *E. coli* cells were homogenized in 5% perchloric acid (PCA), and the acid-soluble fractions were obtained by centrifugation at 18,000 × *g* for 5 min. Supernatants were analyzed in a JASCO HPLC system using a Crestpak C18S column (4.6 by 150 mm) heated to 40°C. Elution was done using a stepwise gradient with solvent A (0.1 M sodium acetate, 10 mM sodium 1-hexanesulfonate [pH 4.5]) and solvent B (methanol). The gradient parameters were as shown in Fig. 1A. The flow rate of the solvents was 1.0 ml/min. Polyamines were detected by fluorescence after mixing the column effluent with an *o*-phthalaldehyde solution containing 0.06% *o*-phthalaldehyde, 0.4% borate buffer (pH 10.5), 0.1% Brij 35, and 12 mM 2-mercaptoethanol at 40°C. Fluorescence was measured at an excitation wavelength of 365 nm and an emission wavelength of 455 nm. Quantification was achieved by determining the peak area of the fluorescence tracings and reference to standard curves prepared using polyamine standard solutions.

Protein assay. The PCA precipitates were dissolved in 0.1 N NaOH, and their protein contents were assayed using the bicinchoninic acid protein assay reagent (Pierce).

RNA preparation. Total RNA extraction from bacteriocytes was performed using TRIzol reagent (Gibco-BRL). To remove chromosomal DNA contamination, RNA samples were treated with DNase I.

Real-time quantitative RT-PCR. Total RNAs were prepared from bacteriocytes as described above, and the cDNAs were synthesized with 1 μg of total RNA, 1 μl of 200 mM dithiothreitol, and 1 μl of 0.2-μg/μl $pd(N)_6$ primer using the First-Strand cDNA synthesis kit (Pharmacia). PCRs were carried out using

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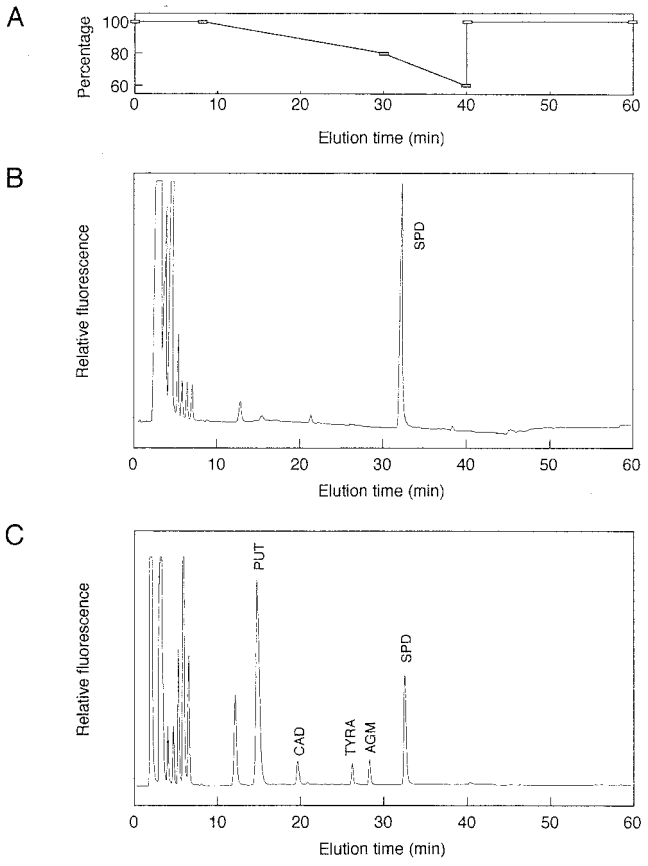

FIG. 1. Analysis of polyamine composition by HPLC. (A) Elution from the reversed-phase column was done using a stepwise gradient with solvent A and solvent B. The gradient parameters are shown as percentages of solvent A. (B) Typical chromatogram of polyamines in *Buchnera*. (C) Typical chromatogram of polyamines in *E. coli*. PUT, putrescine; CAD, cadaverine; TYRA, tyramine; AGM, agmatine; SPD, spermidine.

reverse-transcribed (RT) samples from the preceding step, 0.2 μM (each) target-specific primers (Table 1) (these primers were designed on the basis of sequence data [GenBank accession number AP000398]), 3 mM $MgCl_2$, and LightCycler-DNA Master SYBR Green I (Roche Diagnostics). To prevent the formation of primer dimers, TaqStart antibody (Clontech) was added to the PCR mixture for the hot start. This antibody keeps the DNA polymerase inactive until the temperature rises above 70°C and is inactivated by the same heating step that denatures the target DNA. A LightCycler (Boehringer Mannheim) instrument was used for real-time quantitative PCR. Temperature parameters for PCR amplification were 95°C for 0 s, 55°C for 5 s, and 72°C for 30 s for 40 cycles. The fluorometric intensity of SYBR Green I, a specific dye for double-stranded DNA, was measured at the end of each elongation phase, and a relative amount of each target cDNA was calculated by kinetic analysis (32). Fluorescence signals caused by primer dimers and nonspecific background were discriminated by melting-curve analysis, as recommended by the manufacturer.

TABLE 1. Sequences of the primers used in quantitative RT-PCR

Target gene	Primers	Size of PCR product (bp)
16S rRNA	5′-TGAGACACGGTCCAGACTCCTAC-3′ 5′-GTTGCGCTCGTTGCGGGACTTAAC-3′	790
speD	5′-GCTATGCTAATACCAATGATTCGCG-3′ 5′-TATCTCTAGTAAATCCCCGCACACG-3′	447
speE	5′-GATGATATTGTTCAAACAACTGAACGCG-3′ 5′-GAAGAAAAAAAACACCGTTCTGTGCTAC-3′	445

TABLE 2. Cell size of *Buchnera*[a]

Age of aphid (days)	Cell diam (μm)	Vol (μm^3)
10	2.73 ± 0.11	12.7 ± 1.5
20	2.70 ± 0.10	11.6 ± 1.3
30	2.69 ± 0.07	11.0 ± 0.8
40	2.71 ± 0.07	11.4 ± 1.0
50	2.82 ± 0.06	12.3 ± 0.9

[a] Values are means ± standard errors (n = 50).

RESULTS

Cell size of *Buchnera*. *Buchnera* cells were isolated from about 20 individuals each of 10-, 20-, 30-, 40-, and 50-day-old adult aphids. The volume of *Buchnera* cells did not change significantly as the host aphids aged (Table 2).

Polyamine composition of *Buchnera*. Irrespective of the age of the host aphids, *Buchnera* contained virtually only a single polyamine, spermidine (Fig. 1B). Although putrescine and cadaverine are major polyamines in many prokaryotic organisms, no significant amounts of these polyamines were detected in *Buchnera*. The polyamine composition of *E. coli* was also examined by the same method (Fig. 1C). In *E. coli*, considerable amounts of putrescine, spermidine, cadaverine, agmatine, and tyramine were detected, which was consistent with the previous reports (10, 16, 28).

In Fig. 2, we present changes in the spermidine content in terms of the amount per cell (A), per volume (B), and per milligram of protein (C). In each case, spermidine was the most abundant in *Buchnera* isolated from 10-day-old aphids and amounted to $(1.97 \pm 0.17) \times 10^{-16}$ mol/cell, 16.8 ± 3.2 mM, and 49.6 ± 3.7 nmol/mg of protein. According to the value expressed as the amount per cell, each *Buchnera* cell at day 10 contained about 1.2×10^8 molecules of spermidine, each of which has trivalent positive charges. In the meantime, it has been shown that a *Buchnera* cell in adult aphids contains about 6.4×10^7 bp of DNA (about 100 copies of the genome, whose size is about 640 kb) (17). This suggests that spermidine amounts sufficient to neutralize the total negative charges of phosphates due to the double-stranded DNA in a *Buchnera* cell of young aphids are present.

It is known that a single cell of *E. coli* contains 5.6×10^6 molecules of putrescine and 1.1×10^6 molecules of spermidine (22). As the host aphids grew older, the spermidine content in *Buchnera* decreased (Fig. 2A, B, and C), although the level was still higher than that detected in *E. coli* (1 to 2 mM).

Quantitative RT-PCR of mRNAs for *speD* and *speE*. Total RNAs were extracted from bacteriocytes of 10-, 20-, 30-, 40-, and 50-day-old aphids and reverse transcribed. In the beginning, we quantified 16S rRNA in samples derived from the same amount of total RNAs (Fig. 3A). The amount of 16S rRNA in each age sample was used as an internal standard to calibrate the amounts of mRNAs for *speD* and *speE* (each value obtained was divided by the amount of 16S rRNA). Relative amounts of these mRNAs are shown in Fig. 3B and C. The mRNAs for *speD* and *speE* were the most abundant in the bacteriocytes isolated from 10-day-old aphids and decreased in amount with the age of the host. These results plausibly account for changes in the amount of spermidine estimated in Fig. 2.

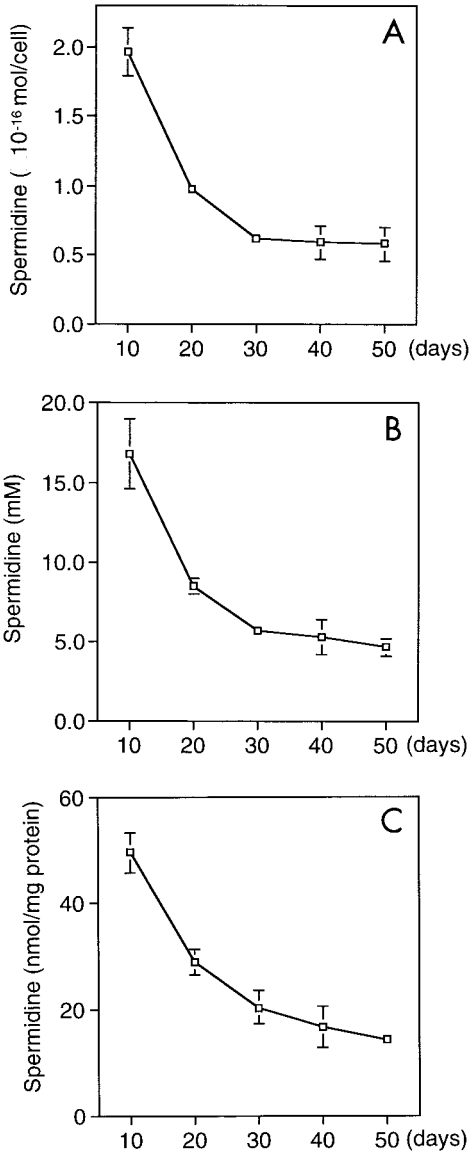

FIG. 2. Spermidine content in *Buchnera*. *Buchnera* cells were isolated from about 20 individuals each of 10-, 20-, 30-, 40-, and 50-day-old adult aphids. Spermidine content is presented in terms of the amount per cell (A), per volume (B), and per milligram of protein (C). Each data point is the mean ± standard error of five replicate groups.

DISCUSSION

The present study revealed that *Buchnera* contained a large amount of only one polyamine, spermidine. This represented a marked difference in the polyamine composition between *Buchnera* and *E. coli*, a bacterium closely related to *Buchnera* (31). In *E. coli*, like most other prokaryotes, the most abundant polyamine is putrescine (10, 16, 28), a divalent amine. Spermidine is a trivalent amine with high affinity to DNA and thus, compared with putrescine, much higher activity to stabilize DNA molecules (5, 24, 25). In this context, it is important to consider the cell size and unique genome structure of *Buchnera*. The cell volume of *Buchnera* (ca. 10 μm^3; Table 2) is about 10 times that of *E. coli* (0.5 to 1.0 μm^3 [19]). Since *Buchnera* has more than 100 copies of the genome (17), whose size is about a seventh of that of the *E. coli* genome (4), the total amount of DNA molecules in a *Buchnera* cell is also about 10 times as great as that of an *E. coli* cell. Therefore, the DNA volume in *Buchnera* is roughly similar to that of *E. coli*, indicating that an extraordinarily large number of circular DNA molecules have to be stabilized in a large *Buchnera* cell. For this reason, it is conceivable that a unique mechanism for dealing with DNA molecules is needed by *Buchnera*. A high concentration of spermidine, which is an efficient stabilizer of DNA, in *Buchnera* can be involved in this mechanism. We also found that the spermidine content decreased with the age of the host aphid. It was demonstrated that the distribution of

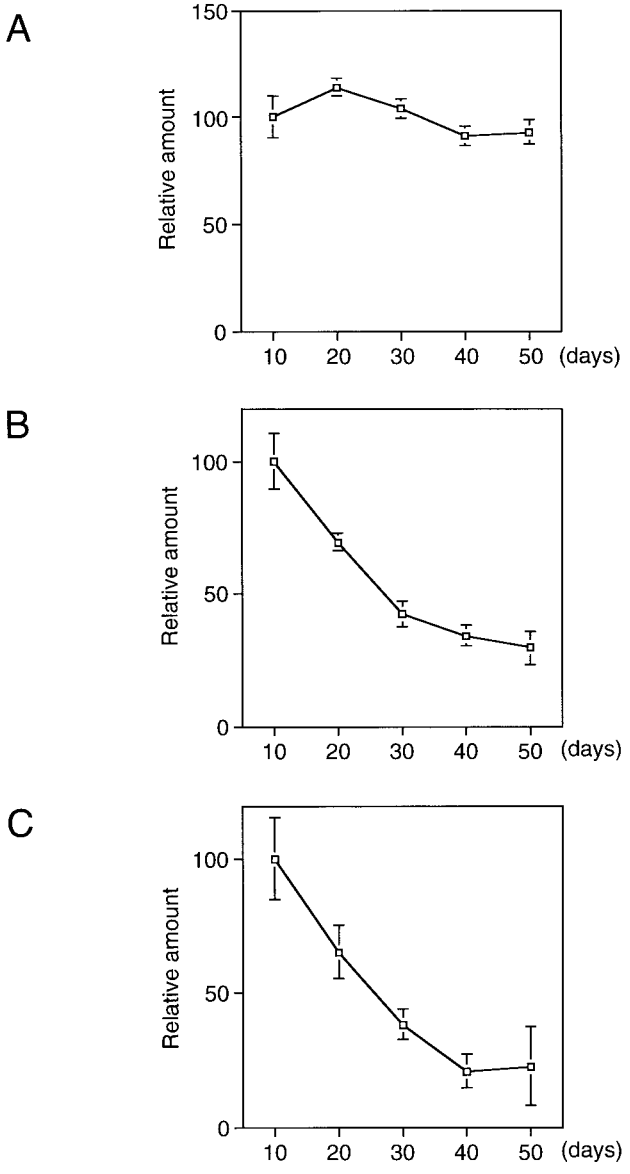

FIG. 3. Relative amounts of *speD* and *speE* mRNAs analyzed by quantitative RT-PCR. Total RNA was prepared from bacteriocytes of aphids at the indicated ages (days). cDNAs were synthesized with $pd(N)_6$ primer using the First-Strand cDNA synthesis kit (Pharmacia) and quantified by PCR amplification with a LightCycler instrument (Boehringer Mannheim). Amounts of target cDNAs in each sample are expressed as a proportion of that in *Buchnera* from 10-day-old aphids. Each data point is the mean ± standard error of five replicate groups. (A) Relative amount of 16S rRNA. The value of each data point was used for calibration in panels B and C. (B) Relative amount of *speD* mRNA. (C) Relative amount of *speE* mRNA.

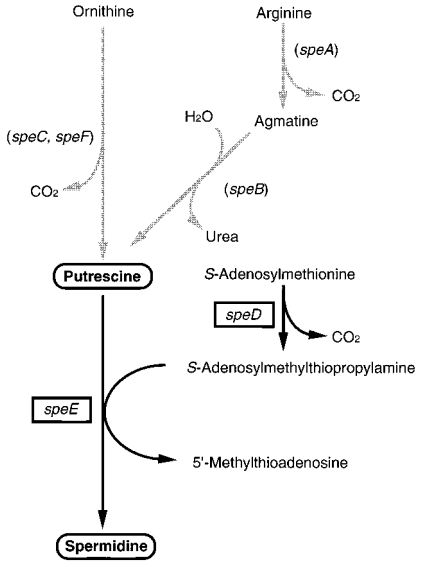

FIG. 4. Biosynthetic pathway of polyamines. In *Buchnera*, the pathways to synthesize putrescine are absent. The enzymes encoded by the genes are as follows: *speA*, arginine decarboxylase (EC 4.1.1.19); *speB*, agmatinase (EC 3.5.3.11); *speC*, ornithine decarboxylase isozyme (EC 4.1.1.17); *speD*, *S*-adenosylmethionine decarboxylase (EC 4.1.1.50); *speE*, spermidine synthase=putrescine aminopropyl transferase (EC 2.5.1.16); *speF*, ornithine decarboxylase, inducible (EC 4.1.1.17). Genes present in the *Buchnera* genome are boxed, while those absent are in parentheses.

DNA in the *Buchnera* cell changes with the age of the host aphid (18). DNA molecules apparently spread uniformly throughout the cell that was isolated from young (18-day) aphids, while the *Buchnera* cells from middle-aged (30-day) or older (40-day) aphids showed heterogeneous distribution of DNA. These findings may support the hypothesis that spermidine is required to stabilize the large number of DNA molecules in *Buchnera* cells. However, this does not necessarily mean that all organisms containing large amounts of spermidine have many genomic copies. Spermidine is known as the major polyamine in *Bacillus subtilis* also, which is a gram-positive bacterium phylogenetically distant from *Buchnera* (12). In the case of *B. subtilis*, spermidine is essential for sporulation, which requires compaction of the genomic DNA into a small specialized cell, the spore.

DNA molecules stabilized by spermidine do not form tight aggregates but form highly fluid liquid crystal structures, which cannot be accomplished by inorganic cations or proteins (24). This fluidity enables DNA-binding proteins to get access to DNA molecules, which is prerequisite to gene expression and DNA replication, although it is uncertain whether all the copies of the *Buchnera* genome function actively. The concentration of spermidine in *Buchnera* was higher when the host aphids were young, suggesting that this polyamine also plays an important role in DNA replication in *Buchnera*, since the genomic copy number of *Buchnera* increases with time when aphids are young (18). This is consistent with the previous reports indicating that an increase in polyamine biosynthesis was required for DNA replication of many other prokaryotic and eukaryotic cells (5, 28).

We examined the expression of the *speD* and *speE* genes in *Buchnera*, whose products are key enzymes in spermidine synthesis. The mRNAs for *speD* and *speE* were the most abundant in *Buchnera* isolated from 10-day-old aphids and decreased with age of the host, which was in line with the change in spermidine content. This finding suggests that spermidine detected in *Buchnera* (Fig. 2) is synthesized through *Buchnera*'s own metabolism. Whole-genome analysis of *Buchnera* revealed that this bacterium has no other genes than *speD* and *speE* that are involved in the polyamine biosynthetic pathway (28), while *E. coli* has six of them, *speA*, *speB*, *speC*, *speD*, *speE*, and *speF* (2) (Fig. 4). In other words, *Buchnera* conserves genes that are essential to synthesize spermidine in spite of a drastic reduction in the genome size, suggesting that spermidine is an indispensable substance for *Buchnera*. However, it is yet to be answered how *Buchnera* produces spermidine without the ability to synthesize its precursors, such as agmatine and putrescine. The most probable scenario is that the host provides *Buchnera* with these precursors. It is already known that *Buchnera* and host aphids exchange amino acids to meet their metabolic requirements (26, 27). Therefore, it is not farfetched to suppose that host aphids affect the physiology of *Buchnera* through controlling the supply of polyamine precursors.

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