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Phosphocarrier Proteins in an Intracellular Symbiotic Bacterium of Aphids[1]

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A GroEL homolog produced by *Buchnera*, an intracellular symbiotic bacterium of aphids, is not only a molecular chaperone but also a novel phosphocarrier protein, suggesting that this protein plays a role in a signal transducing system specific to bacteria living in an intracellular environment. This prompted us to look into phosphocarrier proteins of *Buchnera* that may be shared in common with other bacteria. As a result, no evidence was obtained for the presence of sensor kinases of the two-component system in *Buchnera*, which are found in many bacteria. It is possible that the lack of sensor kinases is compensated for by the mulitifunctional GroEL homolog in this symbiotic bacteria. In contrast, we successfully identified three phosphotransferase system genes, *ptsH*, *ptsI*, and *crr* in *Buchnera*, and provide evidence for their active expression. While the deduced amino acid sequences of these gene products, histidine-containing phosphocarrier protein, Enzyme I, and Enzyme III were similar to their counterparts in *Escherichia coli*, the predicted isoelectric points of the *Buchnera* proteins were strikingly higher. It was also suggested that *Buchnera* Enzyme I, when produced in *E. coli*, is able to accept the phosphoryl group from phosphoenolpyruvate, but not from ATP.

Key words: aphid, phosphorelay, PTS, symbiotic bacterium, two-component system.

It has been revealed that three types of protein-phosphorylating systems mediate signal transduction in bacteria. These are: 1, classical protein kinase/phosphatase systems; 2, sensor-kinase/response-regulator systems, or two-component systems; and 3, phosphoenolpyruvate: sugar phosphotransferase systems (PTS) (*1*). Among the three systems, the latter two share the common characteristic that their protein components transduce signals by the formation of high-energy phosphoamino acids such as phosphohistidine and phosphoaspartate (*2*). In the two-component systems, the sensor kinase, in response to environmental stimuli, phosphorylates its own histidine residue autocatalytically using ATP as a substrate, then transfers the phosphoryl group to an aspartate residue in the response regulator, and thus mediates changes in gene expression (*3*). More than 30 different sensor kinases have been identified in over 30 prokaryotic species as well as in several eukaryotes (*4*). The high sequence conservation and widespread occurrence of these phosphocarrier proteins suggest that they are a highly conserved components for the coordinated regulation of adaptive responses (*5*). PTS mediate the uptake of, concomitant phosphorylation of, and chemotaxis toward a large number of sugars (*6*). It has been demonstrated that the system consists of two cytoplasmic proteins, the histidine-containing phosphocarrier protein (HPr) and Enzyme I, which are required for the transport and phosphorylation of all PTS sugars in common (*7*). In addition, sugar-specific, cytoplasmic and/or membrane proteins, Enzyme II and Enzyme III, are required for the transport of individual PTS sugars (*7*). In the PTS, a histidine residue in Enzyme I accepts the phosphoryl group from phosphoenolpyruvate (PEP) autocatalytically, and a multi-step phosphorelay is initiated by the tranfer of the phosphoryl group to a histidine residue of HPr (*8*).

The bacteriocyte of aphids (Homoptera; Insecta) harbors in its cytoplasm a large number of bacterial symbionts, *Buchnera*, which are closely related phylogenetically to *Escherichia coli* (*9-11*). The symbiosis between aphids and *Buchnera* dates back some 200 million years (*12*), and is obligately mutualistic, representing one of the most intimate relationships between different organisms. *Buchnera* are vertically transmitted through each host generation, and can no longer grow outside the aphid bacteriocyte because of their prolonged intracellular life (*10, 11*). For these intracellular bacteria, sensing signals from the host cytoplasm must be of paramount importance to maintain the integrity of the association. This has prompted us to look into their signal transducing systems. In an effort to characterize symbionin, a GroEL homolog that is selective-

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Abbreviations: aa., amino acid; DIG, digoxigenin; HPr, histidine-containing phosphocarrier protein; nt, nucleotide; PEP, phosphoenolpyruvate; PTS, phosphotransferase system.

ly produced by *Buchnera* in large amounts (*13-15*), we noticed that this protein is able to function not only as a molecular chaperone, similar to GroEL in other bacteria, but also as an energy-coupling phosphocarrier (*16*). Like sensor kinases in the two-component systems, the GroEL homolog mediates the phosphoryl transfer from ATP to other proteins through the autophosphorylation of one of its histidine residues (*17*). Nevertheless, there is no structural similarity between the protein and the sensor kinases known to date, and symbionin also shows no similarity to either HPr or Enzyme I of the PTS, suggesting that this GroEL homolog serves as a novel phosphocarrier of a signal transducing system specific to bacteria living in an intracellular environment (*18*). With this consideration in mind, we have undertaken a molecular approach to identifying energy-coupling phosphocarrier proteins in *Buchnera*.

MATERIALS AND METHODS

Isolation of Buchnera Cells from Aphids—A long-established parthenogenetic clone of pea aphids, *Acyrthosiphon pisum* (Harris), was maintained on young broad bean seedlings at 15°C with 18 h of light and 6 h of darkness (*13*). Young apterous aphids were dissected in isotonic buffer under a microscope, and 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 isotonic buffer and passing the suspension through an isopore membrane with a pore size of 3 μm to remove the nuclei and bacteriocytes (*19*). *Buchnera* cells were collected by centrifugation at 1,500 $\times g$.

DNA Extraction and Southern Hybridization—*Buchnera* cells were treated with lysozyme and proteinase K, and the genomic DNA was extracted according to the standard phenol/chloroform/isoamyl alcohol method. Subsequently, DNA was treated with RNase A, followed by treatment with phenol/chloroform/isoamyl alcohol (*20*). Purified DNA was digested with restriction endonucleases, resolved by agarose gel electrophoresis, and blotted on a nylon membrane (Hybond-N$^+$, Amersham). Hybridizations were performed using DNA segments encoding sensor kinases and HPr of *E. coli* as probes, which had been amplified and labeled with digoxigenin (DIG) using a DIG PCR labeling mixture (Boehringer). Other methods used for prehybridization, hybridization, washing, and detection of signals by chemiluminescence with the DIG detection kit (Boehringer) were performed as instructed by the supplier.

PCR Detection of Relevant Genes—For the detection of genes for the sensor kinases, degenerate primers were constructed according to the nucleotide sequences for conserved motifs in the *E. coli* sensor kinases, such as H-, N-, F-, G1-, and G2-box (*4*). For the detection of genes for HPr (*ptsH*) and Enzyme I (*ptsI*), the following degenerate primers were constructed based on the conserved regions of these genes in several bacteria:

*ptsH*1: 5′-CCNGCNGCNCARTTYGTNAARGA-3′
*ptsH*2: 5′-GCYTTYTYGTYCRTCYTCNCCYTC-3′
*ptsI*1: 5′-GAYYTIACNCCNWSNGARACNGC-3′
*ptsI*2: 5′-ARYTCNCCRCACATNCCNGYCCA-3′

The bases of the primers are denoted according to the IUB codes: I, inosine; R, A or G; Y, C or T; W, A or T; S, G or C; N, A or G or C or T. When PCR products were obtained, they were cloned and sequenced.

Cassette PCR Amplification—*Buchnera* genomic DNA was digested with *Eco*RI and *Sau*3AI, and ligated to relevant cassette DNAs. The DNA segments containing *ptsH* and *ptsI* were amplifed using a TaKaRa LA PCR *in vitro* Cloning Kit and the above cassette-ligated DNA fragments as templates (*21*). PCR products obtained were sequenced directly using an ABI PRISM 310 Genetic Analyzer.

RT-PCR—Total RNA was prepared from *Buchnera* cells using TRIzol reagent (Gibco BRL) and treated with DNase I. Using the total RNA as templates, cDNAs were prepared using a first-strand synthesis kit (Pharmacia). Using these cDNAs as templates with primers specific to each gene, DNA segments of the PTS-related genes were amplified by PCR, and sequenced.

Over Expression and Purification of Enzyme I Fusion Protein—*ptsI* of *Buchnera* was expressed in *E. coli* using the pET-32 EK/LIC vector kit (Novagen). Using BL21-(DE3)pLysS as an expression strain of *E. coli*, Enzyme I was produced as a fusion protein. Transformed cells were cultured in LB, and expression of the fusion gene was induced by the addition of IPTG. A fusion protein containing Enzyme I was purified from the inclusion body formed in the transformed *E. coli* cells using His-Bind Resin (Novagen) under denaturing conditions as instructed by the supplier, and concentrated by passing through a Mol-Cut ultrafiltration membrane, UFP1 TTK24 (Millipore).

Autophosphorylation of Enzyme I—The fusion protein containing Enzyme I was purified as above, and further resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After removing SDS, the gel was incubated with either [^{32}P]PEP or [γ-^{32}P]ATP, washed, and subjected to autoradiography (*22*). [^{32}P]PEP was prepared from [γ-^{32}P]ATP and pyruvate using pyruvate kinase (*23*).

DNA Sequence Analysis—DNA sequences were analyzed using a DNASIS-MAC (Version 3.7, Hitachi). Homology searches were performed using BLAST programs at the National Center for Biotechnology Information server (Bethesda, MA).

RESULTS

Detection of Genes for Phosphocarrier Proteins in Buchnera—Genes for sensor kinases in *Buchnera* were extensively searched by Sourthern blot hybridization using DNA fragments from 8 genes for the *E. coli* sensor kinases, *envZ, ntrB, phoR, narX, cpxA, arcB, basS*, and *uhpB*, as probes. Under all experimetal conditions explored, however, none of these genes gave a positive signal (data not shown). Degenerate primers were constructed based on the

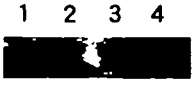

Fig. 1. **Detection of the PTS genes of *Buchnera* by Southern blot hybridization.** *Buchnera* DNA was digested with *Bam*HI (lane 1), *Eco*RI (lane 2), and *Hin*dIII (lane 3), resolved by agarose gel electrophoresis, and blotted on a nylon membrane. Hybridization was performed using the DNA segment encoding HPr of *E. coli*, amplified and labeled with DIG, as a probe. Signals were detected by chemiluminescence due to DIG. In lane 4, *E. coli* DNA was digested with *Eco*RI as a positive control.

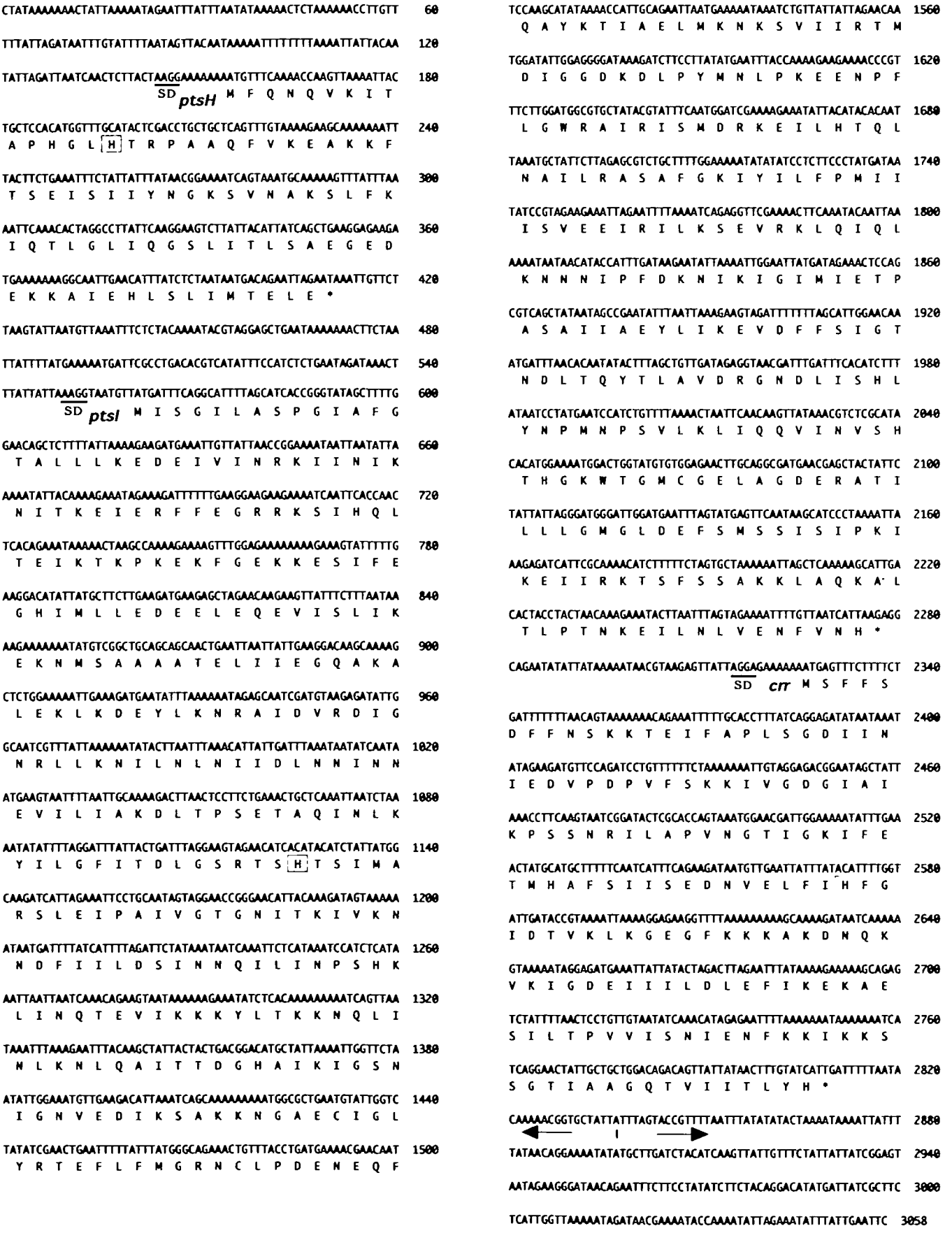

Fig. 2.

conserved sequences for the 5 motifs in the 8 *E. coli* sensor kinases (see in "MATERIALS AND METHODS"), and used in various combinations to detect relevant genes in the *Buchnera* genome by PCR. No combination of these degenerate primers gave PCR products when *Buchnera* DNA was used as a template (data not shown).

Southern blot hybridization of *Buchnera* DNA using the entire sequence of *E. coli ptsH* as a probe suggested the presence of a gene corresponding to *ptsH* in *Buchnera* (Fig. 1). PCRs using degenerate primers constructed based on the conserved sequences of *ptsH* and *ptsI* in various bacteria successfully amplified relevant gene fragments. Subsequently, we further amplified longer sequences containing these *ptsH*- and *ptsI*-related regions by cassette PCR using *Buchnera* DNA as a template. On sequencing these PCR products, it was demonstrated that *ptsI* resides adjacent to and downstream of *ptsH*. In addition, it was noticed that *ccr*, the gene for glucose-specific Enzyme III, is located downstream of *ptsI*. In view of the nucleotide sequences flanking these genes and the intergenic sequences, it is highly likely that *ptsH*, *ptsI* form a single operon (Fig. 2) as in *E. coli*.

Structure of HPr, Enzyme I, and Enzyme III of Buchnera—The amino acid sequences of HPr, Enzyme I, and glucose-specific Enzyme III of *Buchnera* deduced from the corresponding nucleotide sequences are shown in Fig. 2, and a comparison of these proteins with those of *E. coli* is shown in Table I. As marked in Fig. 2, the histidine residues that accept phophoryl moieties are conserved in all these *Buchnera* proteins.

Expression of PTS Genes in Buchnera—cDNAs were prepared using total RNA extracted from *Buchnera* cells as a template. When these cDNAs and *ptsH*-specific sequences were used as templates and primers respectively, PCR amplified a DNA fragment whose size was exactly the same as that obtained when the *Buchnera* genomic DNA was used instead of the cDNAs as a template. The same result was obtained when either *ptsI*- or *ccr*-specific sequences were used as PCR primers (Fig. 3). Sequence analyses demonstrated that these RT-PCR products are partial copies of the three PTS genes (data not shown), showing that the PTS genes are active in *Buchnera* cells.

Autophosphorylation of Enzyme I—*E. coli* was transformed by an expression plasmid vector containing *Buchnera ptsI* as a fusion gene. The fusion protein containing Enzyme I, which localized largely in the inclusion bodies of transformed cells, was successfully recovered using His-Bind Resin and a Mol-Cut ultrafiltration membrane (Fig. 4A). Using the fusion protein purified by SDS-PAGE, autophosphorylation of Enzyme I was examined by the in-gel assay method (*22*). When the gel was incubated with [^{32}P]PEP, a fusion protein containing Enzyme I of *Buch-*

Fig. 2. **Structure of the *ptsH-ptsI-crr* operon of *Buchnera* and deduced amino acid sequences of the encoded polypeptides.** Parts of *ptsH* and *ptsI* of *Buchnera* were amplifed by PCR using the degenerate primers shown in "MATERIALS AND METHODS." Using these gene fragments as primers, the *ptsH-ptsI-crr* operon and its flanking regions were amplified by cassette PCR, and directly sequenced. The boxed H in each polypeptide indicates a histidine residue that corresponds to the phosphorylation site in the PTS of *E. coli*. The pair of horizontal arrows (labeled I) indicates a palindrome that is presumed to be a cue for transcriptional termination. SDs are for presumed Shine-Dalgarno sequences.

TABLE I. **Comparison of PTS proteins of *Buchnera* with those of *E. coli*.**

	HPr		Enzyme I		Enzyme III	
	Buchnera	*E. coli*	*Buchnera*	*E. coli*	*Buchnera*	*E. coli*
Total aa number	85	85	571	575	161	169
Molecular weight	9,411	9,118	64,359	63,558	17,785	18,250
Predicted pI	9.21	5.54	9.22	4.63	7.72	4.58
Sequence identity						
aa	72.9%		66.7%		61.9%	
nt	69.4%		62.8%		61.9%	

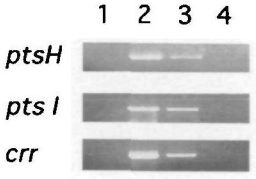

Fig. 3. **Detection of PTS gene transcripts by RT-PCR.** Using the total RNA from *Buchnera* as a template, cDNAs were prepared and subjected to PCR using a primer set specific to *ptsH*, *ptsI*, or *crr* (lane 3). After electrophoresis on agarose, bands were detected with ethidium bromide. Lanes 1, 2, and 3 show none, *Buchnera* DNA, and *Buchnera* RNA treated with DNase I used as templates for PCR, respectively.

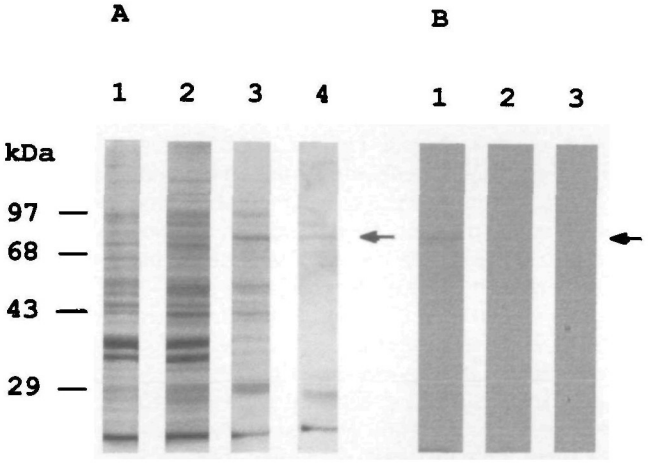

Fig. 4. **Autophosphorylation of Enzyme I.** Panel A, purification of a fusion protein containing Enzyme I of *Buchnera*. *E. coli* cells that had been transformed by *Buchnera ptsI* were cultured in LB, and gene expression was induced by the addition of IPTG. The inclusion body was lysed in buffer containing 6 M urea, and the fusion protein was purified using His-Bind Resin under denaturing conditions. The eluate containing the fusion protein from the resin was passed through a Mol-Cut ultrafiltration membrane. Proteins at each purification step were resolved by SDS-PAGE, and stained with CBB. 1, whole cell lysate of *E. coli* before the addition of IPTG; 2, whole cell lysate of *E. coli* after the addition of IPTG; 3, inclusion body lysate; 4, purified fusion protein. An arrow indicates the position of the fusion protein containing Enzyme I of *Buchnera*. Panel B, determination of autophosphorylation of Enzyme I by in-gel assay (*22*). The fusion protein was separated by SDS-PAGE, and the gel was soaked in an excess volume of Tris-HCl buffer (pH 7.5) to remove SDS. Then, the gel was incubated with either [^{32}P]PEP or [γ-^{32}P]ATP in the same buffer containing 5 mM $MnCl_2$. After the reaction, unreacted radioisotopes were removed by incubating the gel with Dowex 2×8-50 anion-exchange resin. The gel was rinsed and subjected to autoradiography using Kodak X-Omat film. 1, [^{32}P]PEP; 2, [^{32}P]PEP in the presence of a 100-fold molar excess of unlabled PEP; 3, [γ-^{32}P]ATP. The arrow indicates the position of the fusion protein.

nera with a molecular mass of 75 kDa was found to be labeled (Fig. 4B, lane 1). The same experiment performed in the presence of a 100-fold molar excess of unlabled PEP did not give a radioactive band (Fig. 4B, lane 2). When the gel was incubated with [γ-^{32}P]ATP instead of [^{32}P]PEP, again no radioactive band was observed (Fig. 4B, lane 3). These results clearly indicate that Enzyme I of *Buchnera* is capable of the specific autocatalytic acceptance of the phosphoryl group from PEP.

DISCUSSION

Although the two-component system is ubiquitous in many bacteria, and numerous sensor kinases have been identified, in this study we could not detect any sensor kinase genes in *Buchnera* by either Southern blot hybridization or PCR. One possibility is that *Buchnera* have lost the genes for sensor kinases during the course of their prolonged intracellular life. This may be a plausible explanation considering that their genome size is only one-seventh that of *E. coli* (*24*). Indeed, *Mycoplasma* species, which are not only intracellular inhabitants like *Buchnera*, but also have small-sized genomes, have been shown to lack all the genes for the two-component system (*25, 26*). The fact that a GroEL homolog in *Buchnera* has phosphotransferase activity may be relevant to this evolutionary change. It is conceivable that in *Buchnera* the lack of sensor kinases is made up for by the multifunctional chaperone (*17*). However, it should be noted that the lack of sensor kinases is not a general attribute of intracellular bacteria. It has been demonstrated that the intracellular bacterial symbiont of the hydrothermal vent tubeworm, *Riftia pachyptila*, has a sensor kinase (*27*). This obligate symbiont is a sulfide-oxidizing chemoautotrophic bacterium, and, like *Buchnera*, belongs to the gamma subdivision of proteobacteria. For the present, there remains the possibility that *Buchnera* contain a few species of sensor kinase that play roles specific to intracellular life.

In this study, we successfully identified three of the PTS genes of *Buchnera*, and provide evidence for their active expression in this symbiont (Fig. 3). Our findings also suggest that *Buchnera* Enzyme I is able to receive the phosphoryl group from PEP autocatalytically (Fig. 4). Since plant phloem saps, on which aphids feed, are commonly very rich in sugars, it is probable that *Buchnera* retain the PTS in a complete form to utilize the sugars the host ingests. This may account for the conservation of the *ptsH-ptsI-crr* operon in *Buchnera* in spite of a dramatic reduction in their genome size (*24*). In *Mycoplasma*, these PTS genes no longer form an operon, probably as a result of gene rearrangement accompanied by the reduction in genome size (*28*).

The deduced amino acid sequences of HPr, Enzyme I, and Enzyme III were 72.9, 66.7, and 61.6% identical, respectively, to their counterparts in *E. coli* (Table I). Although the conservation of histidine residues in sites corresponding to those in the *E. coli* homologs suggests their functional roles as phosphocarriers, the overall sequence conservation of these proteins is not as high as those of molecular chaperones such as GroEL, GroES, and DnaK (*29, 30*). The most striking difference observed between these phosphocarrier proteins in *E. coli* and *Buchnera* is that the predicted isoelectric points (pIs) are much higher in the *Buchnera* proteins. In particular, for HPr and Enzyme I the difference in pIs is as much as 4.5. Such a large difference in pIs can not be ascribed to amino acid substitutions as a result of the A/T pressure imposed on the *Buchnera* genome (*31*). It is probable that the extraordinarily high pI is the result of molecular adaptation of these phophocarriers to the intracellular environment of *Buchnera*, and that basic amino acid residues have been positively selected for. Relevant to this selection may be the fact that the phosphoramidate bond of histidine is unstable under acidic conditions (*5*). Whether these phosphocarriers with high pIs have acquired any other function in addition to being components of the PTS is a matter of sheer speculation for the present.

The data in Figs. 3 and 4 suggest that the PTS for importing glucose is active in *Buchnera*, although the membrane-bound component, Enzyme II, has not yet been detected. Glucose-specific Enzyme III is not only a necessary component of the PTS for glucose import but also serves as a regulator of adenylate cyclase and the PTS-independent import system for sugars such as lactose (*6*). It is possible that Enzyme III plays these roles in the *Buchnera* cell.

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