JOURNAL OF BACTERIOLOGY, May 2000, p. 2967–2969 Vol. 182, No. 10
0021-9193/00/$04.00+0

Prephenate Dehydratase from the Aphid Endosymbiont (*Buchnera*) Displays Changes in the Regulatory Domain That Suggest Its Desensitization to Inhibition by Phenylalanine

NURIA JIMÉNEZ, FERNANDO GONZÁLEZ-CANDELAS, AND FRANCISCO J. SILVA*

Institut Cavanilles de Biodiversitat i Biologia Evolutiva and Departament de Genètica, Universitat de València, 46071 València, Spain

Received 18 November 1999/Accepted 18 February 2000

***Buchnera aphidicola*, the prokaryotic endosymbiont of aphids, complements dietary deficiencies with the synthesis and provision of several essential amino acids. We have cloned and sequenced a region of the genome of *B. aphidicola* isolated from *Acyrthosiphon pisum* which includes the two-domain *aroQ*/*pheA* gene. This gene encodes the bifunctional chorismate mutase-prephenate dehydratase protein, which plays a central role in L-phenylalanine biosynthesis. Two changes involved in the overproduction of this amino acid have been detected. First, the absence of an attenuator region suggests a constitutive expression of this gene. Second, the regulatory domain of the *Buchnera* prephenate dehydratase shows changes in the ESRP sequence, which is involved in the allosteric binding of phenylalanine and is strongly conserved in prephenate dehydratase proteins from practically all known organisms. These changes suggest the desensitization of the enzyme to inhibition by phenylalanine and would permit the bacterial endosymbiont to overproduce phenylalanine.**

Endosymbiosis is one of the main factors that facilitated the diversification of the major insect groups and their adaptation to a wide variety of ecological niches that would otherwise have been inadequate (8). Aphids are strict phloem-feeders that maintain an endosymbiotic association with *Buchnera aphidicola*, a member of the class *Proteobacteria* (1). The association is obligate for both partners, and it is commonly accepted that the main role of endosymbionts is the provision of essential nutrients to the aphids. However, definitive evidence is rare, and only the provision of the amino acids tryptophan and leucine through the translocation of their biosynthetic genes to plasmids is well documented (2, 6). Phenylalanine seems to be overproduced by the endosymbiont, since lower levels are found in antibiotic-treated aphids (aposymbiotic aphids) than in symbiotic aphids (7). In bacteria, the main phenylalanine biosynthetic pathway starts with chorismate, which is converted to prephenate by the enzyme chorismate mutase (CM; EC 5.4.99.5). This compound is converted to phenylpyruvate by prephenate dehydratase (PDT; EC 4.2.1.51) and later transaminated to phenylalanine (9).

The evolution of the genes encoding CM (*aroQ*) and PDT (*pheA*) in prokaryotic and eukaryotic lineages comprises several duplication and fusion events between them and with other genes. Two of the three major divisions of gram-negative bacteria possess a multienzyme protein (CM/PDT) with the CM and PDT activities. The gene encoding this protein, although frequently denoted *pheA*, should be named *aroQ*/*pheA* in order to show the existence of the two domains (3).

In *Escherichia coli*, one of the closest free-living relatives of *Buchnera*, the biosynthesis of phenylalanine is subjected to gene and feedback enzyme regulation. A feedback inhibition of both CM and PDT activities by phenylalanine has been described (9). This amino acid has been proposed to bind an unknown site in the C-terminal part of the protein where the PDT domain is located (3, 12).

* Corresponding author. Mailing address: Institut Cavanilles de Biodiversitat i Biologia Evolutiva, Universitat de València, Apartat 22085, 46071 Valencia, Spain. E-mail: francisco.silva@uv.es.

In this work, we have compared the *Buchnera aroQ*/*pheA* gene and its encoded protein with those from other organisms, especially *E. coli*, searching for changes that show the adaptation of *Buchnera* to endosymbiosis.

Cloning of the *Buchnera* (*Acyrthosiphon pisum*) *aroQ*/*pheA* gene and flanking regions. Based on the sequence of the *aroQ*/*pheA* gene from enteric bacteria, we designed two degenerate primers in the PDT domain of the gene (PheAd1, 5′-ATCCT CARCCNTTYCARC-3′; and PheAd2, 5′-GTAGAACATYT CYTCCCA-3′). Using total DNA isolated from the aphid *A. pisum*, we amplified by PCR an expected 400-bp fragment which was isolated and sequenced. It was highly similar to *pheA* genes from enteric bacteria, but with a high A+T content (>70%) typical of *Buchnera* genes. A Southern blot with *Buchnera* total DNA helped us to make a restriction map of the region of the bacterial chromosome where the *pheA* gene was placed (Fig. 1) and identified *Eco*RI and *Xba*I as restriction enzymes suitable for the cloning of the complete gene with an inverse PCR strategy. This experiment yielded fragments of around 3.5 and 3.0 kb for *Eco*RI and *Xba*I, respectively.

Structure of the *pheA* genomic region. The DNA region included in the overlapping *Eco*RI and *Xba*I fragments was sequenced. Its 4,371 bp contained four complete genes: *rpsI*, encoding the small subunit ribosomal protein S9; *rplM*, encoding the large subunit ribosomal protein L13; the *aroQ*/*pheA* gene, encoding the bifunctional CM/PDT protein; and the gene *ffh*, encoding the signal recognition particle protein (also called "fifty-four homolog"). In addition, two incomplete genes were present at the ends: the *yhbZ* gene, encoding a hypothetical GTP-binding protein; and the *rpsP* gene, encoding the small subunit ribosomal protein S16 (Fig. 1). Proteins encoded by the genes described above showed the highest similarity to proteins from *E. coli* in all cases, except for the CM/PDT protein, which more closely resembled that of *Erwinia herbicola*.

Comparative analysis of *aroQ*/*pheA* genes of *Buchnera* and *E. coli*. The expression of the *aroQ*/*pheA* genes in *E. coli* and other enteric bacteria is controlled solely by an attenuation system, which includes the sequences containing the stem-loop

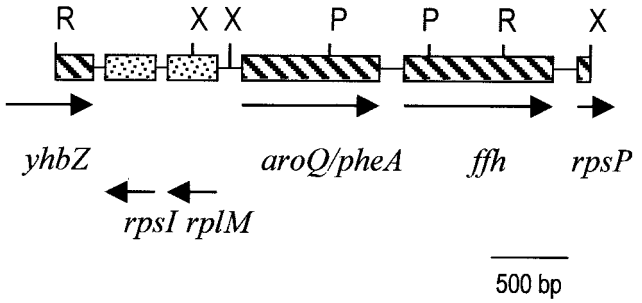

FIG. 1. Genetic map of the 4.4-kb fragment containing the *aroQ/pheA* gene from *Buchnera*. The positions of the *Eco*RI (R), *Pst*I (P), and *Xba*I (X) sites are indicated. Arrows show transcription directions.

structures and a leader region encoding a 15-residue phenylalanine-rich leader peptide (11). The analysis of the 5′ region of the *Buchnera* gene did not show any sequence resembling such an attenuator; hence, adaptation of *Buchnera* to endosymbiosis has probably produced the loss of gene regulation and the change to a constitutive expression to allow for overproduction of phenylalanine.

Comparison of *Buchnera* CM/PDT protein with CM/PDT or PDT proteins from other organisms. The alignment of CM/PDT from *Buchnera* and several other species showed that some parts of the amino acid sequence were well conserved. However, an important feature of *Buchnera* protein was the lack of conservation of the four-residue sequence (ESRP) located in the regulatory part of the PDT domain (Fig. 2). The homologous residues in *Buchnera* were TSQK (residues 329 to 332). When the alignment was extended to monofunctional and trifunctional PDT proteins, the importance of this region was reinforced, since the last two amino acids were conserved in all available sequences, and the first two were conserved in practically all of them. This suggests that the *Buchnera* enzyme could have changed some of its regulatory properties to adapt to the endosymbiotic way of life. Based on the following three arguments, we propose that these changes have produced the desensitization of the enzyme to the inhibitory effect of Phe. Our hypothesis implies that the ESRP sequence is part of the allosteric site of the enzyme. First, in *E. coli*, this sequence is placed in the vicinity of W338, and fluorescent assays have shown that the allosteric binding of Phe takes place close to this amino acid (12). Second, when the regulatory PDT domain was probed against the protein database, a significant similarity was found with the regulatory domain of metazoan aromatic amino acid hydroxylases. In at least two proteins of this family, rat and human phenylalanine hydroxylases (PAHs), it is known that Phe binds the regulatory domain, producing a conforma-

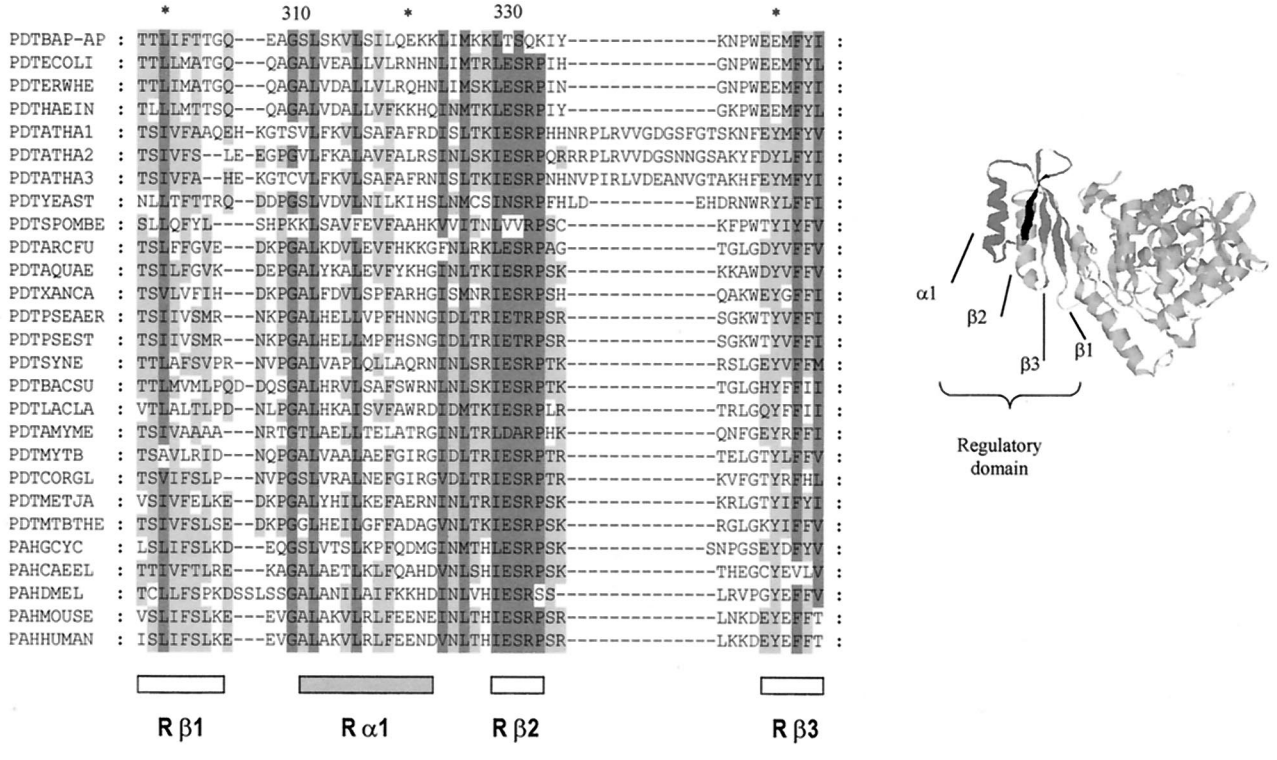

FIG. 2. Alignment of the homologous region of the regulatory domains from PDT and PAH proteins. α-Helix and β-strand positions correspond to the information obtained from the crystal structure of rat PAH (5) (shown to the right). The sequences used in this alignment were as follows (sources and accession numbers are given in parentheses): PDTBAP-AP (*B. aphidicola* [*A. pisum*], AJ239043), PDTECOLI (*E. coli*, P07022), PDTERWHE (*E. herbicola*, Q02286), PDTHAEIN (*Haemophilus influenzae*, P43900), PDTATHA1 (*Arabidopsis thaliana*, O22241), PDTATHA2 (*A. thaliana*, AAD30242), PDTATHA3 (*A. thaliana*, AAC73018), PDTYEAST (*Saccharomyces cerevisiae*, P32452), PDTSPOMBE (*Schizosaccharomyces pombe*, O14361), PDTARCFU (*Archaeoglobus fulgidus*, O30012), PDTAQUAE (*Aquifex aeolicus*, O67085), PDTXANCA (*Xanthomonas campestris*, O87954), PDTPSEAER (*P. aeruginosa*, *Pseudomonas* Genome Project), PDTPSEST (*P. stutzeri*, P27603), PDTSYNE (*Synechocystis* sp., P72808), PDTBACSU (*Bacillus subtilis*, P21203), PDTLACLA (*Lactococcus lactis*, P43909), PDTAMYME (*Amycolatopsis methanolica*, Q44104), PDTMYTB (*Mycobacterium tuberculosis*, P96240), PDTCORGL (*Corynebacterium glutamicum*, P10341), PDTMETJA (*Methanococcus jannaschii*, Q58054), PDTMTBTHE (*Methanobacterium thermoautotrophicum*, 027288), PAHGCYC (*Geodia cydonium*, Y16353), PAHCAEEL (*Caenorhabditis elegans*, CAA91286), PAHDMEL (*Drosophila melanogaster*, Q27599), PAHMOUSE (*Mus musculus*, P16331), PAHHUMAN (*Homo sapiens*, P00439). The numbers at the top indicate the residue position in *Buchnera*.

tional change that activates the protein (4). The presence of the ESRP motif in these proteins (Fig. 2) points to the involvement of this sequence in Phe binding. Besides, the crystal structure of a dimeric rat PAH, which includes the regulatory domain, has been recently reported (5). These authors raise the possibility that the regulatory Phe binding site was located near the interface between the regulatory and catalytic domains in the vicinity of the β-strand Rβ2 (Fig. 2). Third, it has been shown recently (10) that *E. coli* CM/PDT proteins with a change in either E329A, S330A, or R331A produced to different extents a strong increase in the concentration of Phe required to produce a 50% enzyme inhibition and a decrease in Phe binding capacity (less than 10% of the level of wild-type protein).

All of these arguments support the hypothesis that *Buchnera* PDT is not inhibited by phenylalanine, at least to the same extent as in *Buchnera*'s free-living relatives, due to the changes in its regulatory domain described above. Since *Buchnera* proteins are refractory to be active in *E. coli* systems (P. Baumann, personal communication), demonstration of this hypothesis might be best achieved by performing inhibition tests with genetically engineered *E. coli* proteins with amino acid changes mimicking those observed in *Buchnera*, which are currently under way in our laboratory.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been deposited in the GenBank/EMBL database under accession no. AJ239043.

We thank A. Latorre and B. Sabater for helpful comments and suggestions. We are indebted to the Servei de Bioinformàtica and the Servei de Seqüenciació de ADN i proteïnes (S.C.S.I.E., Universitat de València) for computer and technical support.

This work was supported by grant PB96-0793 C04-01 from Dirección General de Enseñanza Superior (Spain).

REFERENCES

1. **Baumann, P., L. Baumann, C.-Y. Lai, and D. Rouhbakhsh.** 1995. Genetics, physiology, and evolutionary relationships of the genus *Buchnera*: intracellular symbionts of aphids. Annu. Rev. Microbiol. **49:**55–94.
2. **Bracho, A. M., D. Martinez-Torres, A. Moya, and A. Latorre.** 1995. Discovery and molecular characterization of a plasmid localized in *Buchnera sp.* bacterial endosymbiont of the aphid *Rhopalosiphum padi*. J. Mol. Evol. **41:**67–73.
3. **Gu, W., D. S. Williams, H. C. Aldrich, G. Xie, D. W. Gabriel, and R. A. Jensen.** 1997. The AroQ and PheA domains of the bifunctional P-protein from *Xanthomonas campestris* in a context of genomic comparison. Microb. Comp. Genomics **2:**141–158.
4. **Hufton, S. E., I. G. Jennings, and R. G. H. Cotton.** 1995. Structure and function of the aromatic amino acid hydroxylases. Biochem. J. **311:**353–366.
5. **Kobe, B., I. G. Jennings, C. M. House, B. J. Michell, K. E. Goodwill, B. D. Santarsiero, R. C. Stevens, R. G. H. Cotton, and B. E. Kemp.** 1999. Structural basis of autoregulation of phenylalanine hydroxylase. Nat. Struct. Biol. **6:**442–448.
6. **Lai, C.-Y., L. Baumann, and P. Baumann.** 1994. Amplification of *trpEG*: adaptation of *Buchnera aphidicola* to an endosymbiotic association with aphids. Proc. Natl. Acad. Sci. USA **91:**3819–3823.
7. **Liadouze, I., G. Febvay, J. Guillaud, and G. Bonnot.** 1995. Effect of diet on the free amino acid pools of symbiotic and aposymbiotic pea aphids *Acyrthosiphon pisum*. J. Insect Physiol. **41:**33–40.
8. **Moran, N. A., and A. Telang.** 1998. Bacteriocyte associated symbionts of insects. Bioscience **48:**295–304.
9. **Pittard, A. J.** 1996. Biosynthesis of the aromatic amino acids, p. 458–484. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed., vol. 1. ASM Press, Washington, D.C.
10. **Pohnert, G., S. Zhang, A. Husain, D. B. Wilson, and B. Ganem.** 1999. Regulation of phenylalanine biosynthesis. Studies on the mechanism of phenylalanine binding and feedback inhibition in the *Escherichia coli* P-protein. Biochemistry **38:**12212–12217.
11. **Xia, T., G. Zhao, and R. A. Jensen.** 1993. The *pheA*/*tyrA*/*aroF* region from *Erwinia herbicola*: an emerging comparative basis for analysis of gene organization and regulation in enteric bacteria. J. Mol. Evol. **36:**107–120.
12. **Zhang, S., G. Pohnert, P. Kongsaeree, D. B. Wilson, J. Clardy, and B. Ganem.** 1998. Chorismate mutase-prephenate dehydratase from *Escherichia coli*. Study of catalytic and regulatory domains using genetically engineered proteins. J. Biol. Chem. **273:**6248–6253.