Applied and Environmental Microbiology, Dec. 1995, p. 4484–4486 Vol. 61, No. 12
0099-2240/95/$04.00+0
Copyright © 1995, American Society for Microbiology

Cloning, Sequencing, and Expression in *Escherichia coli* of the *Bacillus pumilus* Gene for Ferulic Acid Decarboxylase

ANNA ZAGO, GIULIANO DEGRASSI, AND CARLO V. BRUSCHI*

Microbiology Group, International Centre for Genetic Engineering and Biotechnology, AREA Science Park, I-34012 Trieste, Italy

Received 30 May 1995/Accepted 22 September 1995

The *Bacillus pumilus* gene encoding a ferulic acid decarboxylase (*fdc*) was identified and isolated by its ability to promote ferulic acid decarboxylation in *Escherichia coli* DH5α. The DNA sequence of the *fdc* gene was determined, and the recombinant enzyme produced in *E. coli* was purified and characterized.

Phenolic acids are common constituents of forage fed to ruminants. Particularly, ferulic acid and *p*-coumaric acids, mainly in their *trans* configuration, are abundant in graminaceous plants, which represent a major component of ruminant diets. In plant cell walls, these two phenolic compounds bind the complex lignin polymer to the hemicellulose and cellulose (16, 23). It has been suggested that these phenolic acids are released during ruminal degradation of plant cell walls (1). Since these compounds are inhibitory to ruminal microorganisms in vitro (2, 6, 7) and putatively inhibit the digestion of plant cell walls in the rumen by these microorganisms, they are considered an antiquality factor in the forage. Therefore, the modifications of ferulic and *p*-coumaric acids by rumen microorganisms (11) can have an important antitoxicity role.

We focused our attention on the ability of *Bacillus pumilus* PS231, repeatedly isolated from the rumen fluid of a fistulated cow (24) fed with barley straw, to decarboxylate ferulic acid and *p*-coumaric acid to 4-vinylguaiacol and 4-vinylphenol, respectively. This biotransformation not only could be important to improve the ruminal digestive function but also could be of interest as a means of generating value-added chemicals such as guaiacol derivatives from these two aromatic compounds widely distributed in plants.

The decarboxylation of ferulic acids has already been described for several microorganisms (3, 4, 8, 12, 13, 17), and the enzyme has been purified from *Pseudomonas fluorescens* (14) and in our laboratory from *B. pumilus* (9). However, the genes encoding these functions have not been investigated.

In this paper, we describe the identification and isolation of the *B. pumilus trans*-ferulic and *p*-coumaric acid decarboxylase gene, the nucleotide sequence of this gene, and its expression in *Escherichia coli*.

Cloning of *trans*-ferulic acid decarboxylase gene. On the basis of the N-terminal amino acid sequence of the *trans*-ferulic acid decarboxylase previously purified from *B. pumilus* (9), an oligonucleotide probe was synthesized to identify by Southern blot analysis the *fdc* gene in DNA restriction fragments from the same microorganism. The 51-mer oligonucleotide probe was designed by use of the program BackTranslate (Genetics Computer Group, University of Wisconsin) with the codon frequencies of *Bacillus subtilis* (105 genes found in GenBank 63). A Southern blot of *B. pumilus* genomic DNA (5) digested to completion with various restriction enzymes was hybridized at 45°C as described by Drmanac et al. (10) with the ^{32}P-labelled probe. Hybridization with *Hin*dIII digestion displayed a well defined band of approximately 4 kb (data not shown). As a negative control, the probe did not hybridize with total DNA extracted from *E. coli* DH5α. *Hin*dIII DNA fragments ranging between 3.5 and 4.5 kb were purified from agarose with a Qiaex kit (Qiagen, Chatsworth, Calif.) and cloned into the *Hin*dIII site in the polylinker of the plasmid pUC19 to construct a sublibrary (21). This reaction mixture was transformed into *E. coli* DH5α, and transformants were screened by colony hybridization (10). A positive clone containing a 3,690-bp *Hin*dIII fragment was identified. This clone was tested for its ability to decarboxylate ferulic acid in vivo by UV spectroscopy. A culture in Luria-Bertani broth containing 0.5 g of ferulic acid per liter was monitored for the disappearance of this substrate at its maximal absorbance wavelength (310 nm) and the appearance of a new peak at 258 nm (4-vinylguaiacol) (9). Since this fragment conferred to the host strain the ability to decarboxylate *trans*-ferulic acid, we assumed that it contained the entire gene encoding the *trans*-ferulic acid decarboxylase.

Deletion analysis and DNA sequence. To determine which portion of the 3,690-bp *Hin*dIII fragment was required for the conversion of *trans*-ferulic acid to 4-vinylguaiacol, serial deletion plasmids were constructed with *Cla*I, *Eco*RI, and *Xba*I restriction enzymes. These deletion plasmids were introduced into *E. coli* DH5α, and transformed clones were subjected to an in vivo assay for ferulic acid decarboxylase activity, as described above. Figure 1 summarizes the results of the deletion analysis. The 1,332-bp *Hin*dIII-*Xba*I fragment contained in the deletion plasmid pUCFDCΔ_2 encoded all the information necessary for the expression of the decarboxylase activity. This fragment and 347 bases downstream from the unique *Xba*I site in the 3,690-bp insert of pUCFDC were sequenced on both strands by walking primers (ICGEB oligonucleotide synthesizer service) with the Sequenase 2.0 DNA sequencing kit (U.S. Biochemicals, Cleveland, Ohio). A 486-bp open reading frame, starting at an ATG codon at nucleotide 816 and extending to a TAG codon at nucleotide 1301, was found. It encodes a deduced 19,069-Da polypeptide that consists of 161 amino acid residues (Fig. 2).

The nucleotide sequence at the 5′ end of this open reading frame shows high homology with the oligonucleotide used for screening the sublibrary. Moreover, the N-terminal amino acid sequence of the deduced polypeptide is consistent with that obtained by stepwise Edman degradation of the ferulate decarboxylase purified from *B. pumilus* (data not shown). The only difference is a Trp-17 instead of a Val-17. A comparative

* Corresponding author. Mailing address: Microbiology Group, ICGEB, Area Science Park, Padriciano 99, I-34012 Trieste, Italy. Phone: 39 40 375 7304. Fax: 39 40 375 7343. Electronic mail address: bruschi@icgeb.trieste.it.

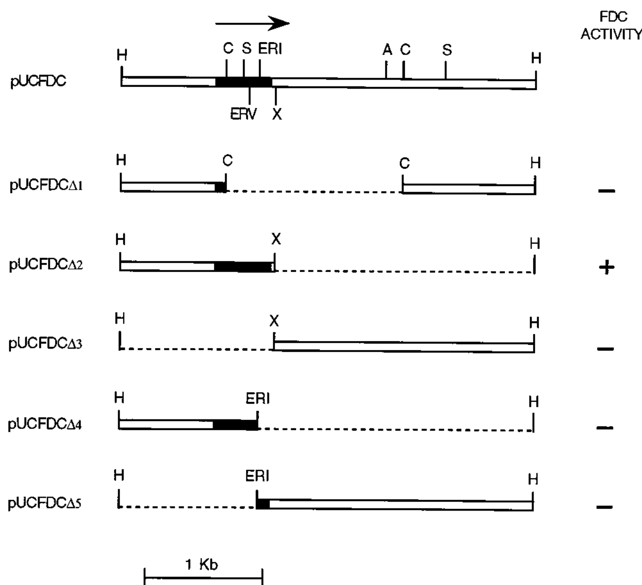

FIG. 1. Restriction enzyme map of the 3,690-bp *Hin*dIII fragment inserted into pUC19 (pUCFDC). Abbreviations: A, *Ava*I; C, *Cla*I; ERI, *Eco*RI; ERV, *Eco*RV; H, *Hin*dIII; S, *Sph*I; X, *Xba*I. The deletion plasmids pUCFDCΔ_1, pUCFDCΔ_2, pUCFDCΔ_3, pUCFDCΔ_4, and pUCFDCΔ_5, made with *Cla*I, *Xba*I, and *Eco*RI, are shown. On the right, the ferulate decarboxylase (FDC) activities of the various transformants with the deletion plasmids are reported.

search with the nucleotide sequence of the *fdc* gene in the GenBank and EMBL data banks as well as with the deduced polypeptide in the SwissProt, PIR, and GenBank data banks by the BLAST mail-server program did not show any interesting homology. The yeast phenylacrylic acid decarboxylase (PAD1), which decarboxylates ferulic acid (8) (GenBank accession number P33751), shows only a 66.7% identity in a 6-amino-acid overlap with the *B. pumilus* ferulate decarboxylase.

A putative Shine-Dalgarno sequence was observed 5 bp upstream from the initiation codon ATG. A potential promoter sequence, TAAAAT, which is similar to the consensus −10 prokaryotic promoter TATAAT, was found between nucleotides 750 and 755. Upstream lies an *E. coli* 35 consensus sequence, TTGACA. The spacing between the −10 and −35 elements is 18 bp, near the optimal 17-base distance (19). Thirty-nine base pairs downstream from the termination codon, there is a sequence resembling a rho-independent terminator. A 645-bp open reading frame of unknown significance was observed on the complementary DNA strand in the opposite orientation, starting 27 nucleotides 5′ from the ATG of *fdc*.

Purification and characterization of recombinant ferulic acid decarboxylase. Ferulic acid decarboxylase (Fdc) expressed in *E. coli* was purified from 8 liters of culture. Cells were collected when the degradation of the substrate was about 50% and lysed as described by Penalva and Salas (20). The protein was purified from the clarified extract by the previously described method (9) with some modifications. A single Q Sepharose fast-flow step was used, followed by a phenyl-Sepharose hydrophobic-interaction chromatography step and a single Sephacryl HR200 final step. The purity of the decarboxylase preparation was demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 3A) performed by the meth-od of Laemmli (15). The overall enzyme yield was 4.3%, with a 205.6-fold purification. The specific activity of the purified enzyme for the decarboxylation of *trans*-ferulic acid was 88 μmol mg^{-1} min^{-1}. The *B. pumilus* ferulic acid decarboxylase expressed in *E. coli* was observed as a ca. 21.5-kDa band in SDS-polyacrylamide gels (versus the theoretical value of 19 kDa). The apparent molecular mass of the native enzyme measured by gel filtration was ca. 42 kDa, indicating that the active *E. coli* enzyme is a homodimer like the enzyme expressed in *B. pumilus* (9). The isoelectric focusing of the purified recombinant decarboxylase, performed with an Ampholine PAGplate precast polyacrylamide gel (Pharmacia Biotech AB, Uppsala, Sweden) with pH values ranging from 3 to 10, showed a major band at pH 4.8 (Fig. 3B) and two very faint bands at a lower pH, one of which corresponded to the isoelectric point 4.6 shown by the native *B. pumilus* decarboxylase (9). Since *B. subtilis* codon usage, which is the only available for *Bacillus* spp., is different from that of *E. coli* (18), it could be inferred that the observed isoforms were due to amino acid misincorporation (22). Alternatively, chemical modifications that occurred during the purification steps could produce isoforms of the enzyme. Nevertheless, these different Fdc species were not detected by high-performance liquid chromatography analysis, which shows only one peak (data not shown). The hydropathy profile determined for the predicted amino acid sequence predicts the ferulate decarboxylase to be a highly hydrophilic and probably cytoplasmic protein. Moreover, the protein has not been found in the culture medium, and no secretion sequences have been identified in the gene. The pI values, the relative dimensions, and the comparison of the N-terminal amino acid sequences suggest that the two

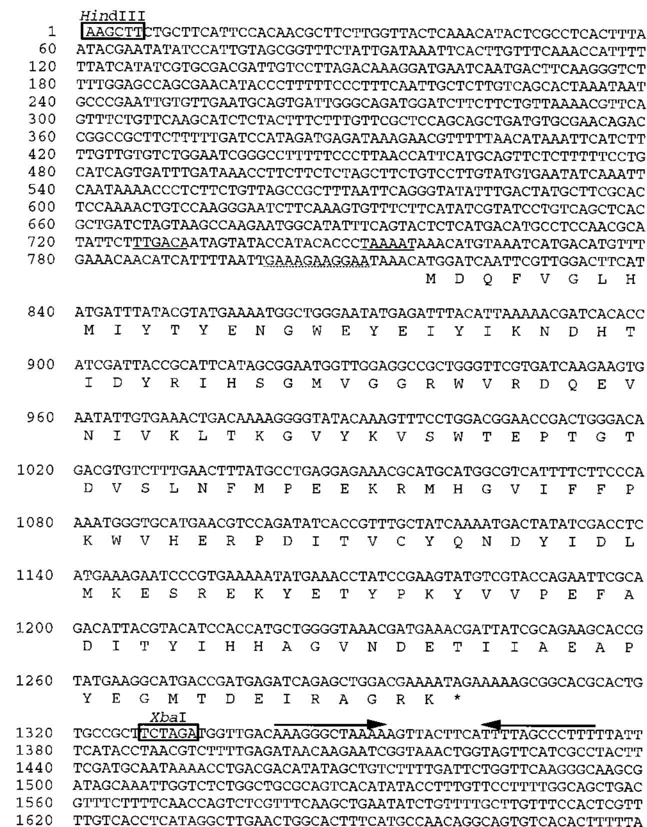

FIG. 2. Nucleotide sequence of the *fdc* gene. Nucleotides are numbered from the 5′ end, and the predicted amino acid sequence for the *fdc* gene is given in single-letter code below the DNA sequence. A possible promoter sequence is underlined; a putative Shine-Dalgarno region is indicated (underlined with a dotted line). Opposing arrows indicate inverted repeats of a possible rho-independent terminator.

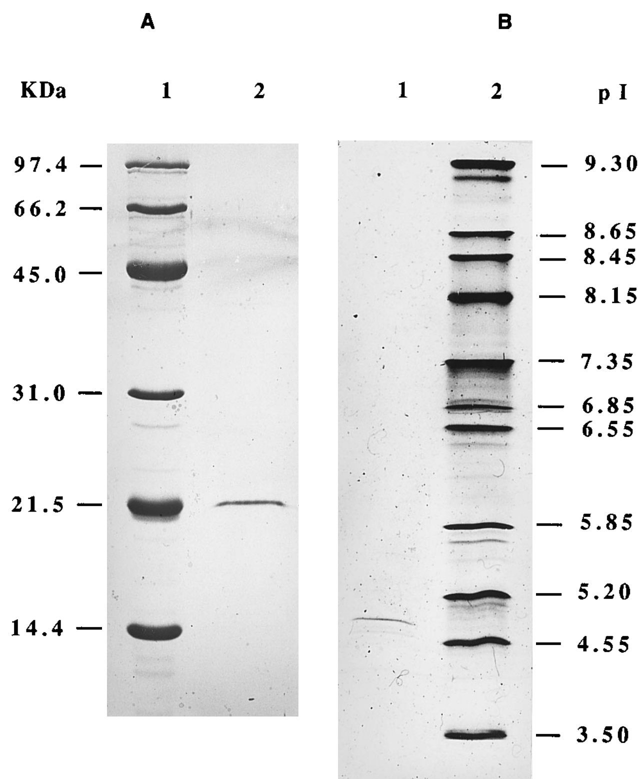

FIG. 3. (A) SDS-PAGE of the ferulate decarboxylase purified from *E. coli*. Lanes: 1, molecular mass standards; 2, peak fraction from the Sephacryl HR200 column. (B) Analytical isoelectric focusing assay of the peak fraction from a Sephacryl HR200 column on a polyacrylamide gel. Lanes: 1, purified recombinant ferulate decarboxylase; 2, pI markers.

proteins from *B. pumilus* and *E. coli* are very similar. However, the ferulate decarboxylase expressed in *E. coli* seems relatively unstable, since a large part of the activity was lost during purification.

In this work, we presented the first isolation of a bacterial gene encoding a ferulate decarboxylase. This activity could be a potential step in the conversion of natural aromatic acids to industrial aromatic intermediates. Since the *fdc* gene is induced by the substrate in *B. pumilus* (9), we will investigate its regulation to optimize its expression. The identification of similar genes not only in *Bacillus* species but also in gram-negative bacteria could also be of interest from a comparative point of view.

Nucleotide sequence accession number. The sequence of the DNA fragment containing the *fdc* gene has been deposited in the GenBank database under accession number X84815.

We gratefully acknowledge P. Zaccaria for his help in computer analysis and G. Ljubijankic for helpful discussion. We thank P. Polverino de Laureto for technical assistance in determining the N-terminal amino acid sequence of the protein.

REFERENCES

1. **Akin, D. E.** 1988. Biological structure of lignocellulose and its degradation in the rumen. Anim. Food. Sci. Technol. **21:**295–310.
2. **Akin, D. E., W. S. Borneman, L. L. Rigsby, and S. A. Martin.** 1993. *p*-Coumaroyl and feruloyl arabinoxylans from plant cell walls as substrates for ruminal bacteria. Appl. Environ. Microbiol. **59:**644–652.
3. **Andreoni, V., E. Galli, and G. Galliani.** 1984. Metabolism of ferulic acid by a facultatively anaerobic strain of *Pseudomonas cepacia*. Syst. Appl. Microbiol. **5:**299–304.
4. **Arfman, H. A., and W. R. Abraham.** 1989. Microbial formation of substituted styrenes. Z. Naturforsch. **44c:**765–770.
5. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1988. Current protocols in molecular biology. Greene Publishing, New York.
6. **Borneman, W. S., D. E. Akin, and W. P. VanEseltine.** 1986. Effect of phenolic monomers on ruminal bacteria. Appl. Environ. Microbiol. **52:**1331–1339.
7. **Chesson, A., C. S. Stewart, and R. J. Wallace.** 1982. Influence of plant phenolic acids on growth and cellulolytic activity of rumen bacteria. Appl. Environ. Microbiol. **44:**597–603.
8. **Clausen, M., C. J. Lamb, R. Megnet, and P. W. Doerner.** 1993. *PAD1* encodes phenylacrylic acid decarboxylase which confers resistance to cinnamic acid in *Saccharomyces cerevisiae*. Gene **137:**107–112.
9. **Degrassi, G., P. Polverino de Laureto, and C. V. Bruschi.** 1995. Purification and characterization of a ferulic acid decarboxylase from *Bacillus pumilus*. Appl. Environ. Microbiol. **61:**326–332.
10. **Drmanac, R., Z. Strezoska, I. Labat, S. Drmanac, and R. Crkvenjakov.** 1990. Reliable hybridization of oligonucleotides as short as six nucleotides. DNA Cell Biol. **9:**527–534.
11. **Healey, J. B., L. Y. Young, and M. Reinhard.** 1980. Methanogenic decomposition of ferulic acid, a model lignin derivative. Appl. Environ. Microbiol. **39:**436–444.
12. **Huang, Z., L. Dostal, and J. P. N. Rosazza.** 1993. Mechanisms of ferulic acid conversions to vanillic acid and guaiacol by *Rodotorula rubra*. Appl. J. Biol. Chem. **268:**23950–23958.
13. **Huang, Z., L. Dostal, and J. P. N. Rosazza.** 1993. Microbial transformation of ferulic acid by *Saccharomyces cerevisiae* and *Pseudomonas fluorescens*. Appl. Environ. Microbiol. **59:**2244–2250.
14. **Huang, Z., L. Dostal, and J. P. N. Rosazza.** 1994. Purification and characterization of a ferulic acid decarboxylase from *Pseudomonas fluorescens*. J. Bacteriol. **176:**5912–5918.
15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
16. **Muller-Harvey, I., and R. D. Hartley.** 1986. Linkage of *p*-coumaroyl and feruloyl groups to cell-wall polysaccharides of barley straw. Carbohydr. Res. **148:**71–85.
17. **Nazareth, S., and S. Mavinkurve.** 1986. Degradation of ferulic acid via 4-vinylguaiacol by *Fusarium solani*. Can. J. Microbiol. **32:**494–497.
18. **Ogasawara, N.** 1985. Markedly unbiased codon usage in *Bacillus subtilis*. Gene **40:**145–150.
19. **Oliphant, A. R., and K. Struhl.** 1988. Defining the consensus sequences of *E. coli* promoter elements by random selection. Nucleic Acids Res. **16:**7673–7683.
20. **Penalva, M. A., and M. Salas.** 1982. Initiation of phage phi29 DNA replication in vitro: formation of a covalent duplex between the terminal protein, p3, and 5′-dAMP. Proc. Natl. Acad. Sci. USA **79:**5522–5526.
21. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Scorer, C. A., M. J. Carrier, and R. F. Rosenberg.** 1991. Amino acid misincorporation during high-level expression of mouse epidermal growth factor in *Escherichia coli*. Nucleic Acids Res. **19:**3511–3516.
23. **Smith, M. M., and R. D. Hartley.** 1983. Occurrence and nature of ferulic acid substitution of cell-wall polysaccharides in graminaceous plants. Carbohydr. Res. **118:**65–80.
24. **Susmel, P., B. Stefanon, C. R. Mills, and M. Colitti.** 1989. The evaluation of PDI concentrations in some ruminant feedstuff: a comparison of *in situ* and *in vitro* protein degradability. Ann. Zootechnol. **38:**269–283.