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Ligninolytic Enzymes in the Ruminal Ecosystem: Molecular Analysis of the Bacterial Ferulic Acid Decarboxylase Gene and its Expression in Yeast

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CONTENTS

SUMMARY

The aim of this work was the identification and characterization of novel microbial enzymatic activities involved in lignin degradation and the isolation of their corresponding genes. The rumen is one of the environments where lignin seems to be degraded, owing to the presence of bacteria and fungi described as potential utilizers of lignin or its constituents. For these reasons this "biofermentor" was chosen as a model for our investigation. Rumen microorganisms showed ability to modify preferentially *trans*-ferulic and *p*-coumaric acid. These phenolic acids are very important in the structure and stability of lignocellulose since they form a bridge between the lignin aromatic biopolymer and cell wall polysaccharides. They are the main aromatic compounds released from the plant cell wall during lignocellulose mineralization and they could represent an interesting renewable source for the industrial production of value-added aromatic substances.

The enzyme which seems to be responsible for the modification of ferulic and *p*-coumaric acid in a wide range of rumen bacteria, and particularly in *Bacillus* species, was identified and purified in our laboratory from a *Bacillus pumilus* strain isolated from cow rumen fluid. This activity has been demonstrated to be a decarboxylase which is able to modify ferulic acid to 4-vinylguaiacol and *p*-coumaric acid to 4-vinylphenol, is inducible by the substrates and is specific for these two cinnamic compounds.

The *B. pumilus* ferulate decarboxylase gene (*fdc*) was identified and isolated by its ability to promote ferulic and *p*-coumaric acid decarboxylation in *Escherichia coli* DH5α. The DNA sequence of the gene was determined and the recombinant enzyme produced in *E. coli* was purified and characterized. The deduced aminoacid sequence did not show homology to any known protein.

This gene was expressed in a *Saccharomyces cerevisiae* strain under the control of yeast regulatory sequences and its product was more active toward ferulic and *p*-coumaric acid than the endogenous phenylacrylic acid decarboxylase of the host.

The regulation of the *fdc* gene as well as the metabolic role of this decarboxylase function, are interesting questions which warrant further investigation.

1. INTRODUCTION

The majority of biomass on Earth is in the form of lignocellulose produced via plant photosynthesis and deposited in the plant cell wall. Several technological processes could gain from an improved understanding of the nature of lignocellulose and how it might be utilized more effectively. These include manufacture of pulp and paper, production of animal feed from straw and cropped grasses, preparation of mushrooms compost and the disposal of urban organic waste.

Biotechnological use of lignocellulosic materials is a very difficult task and the transfer of this technology has not progressed as rapidly as one would have desired. One reason for this was a lack of basic knowledge of the biodegradative processes and the enzymes involved. In this respect, the study of the fundamental problem of lignocellulose breakdown, including biological processes, would be beneficial. If the major components, lignin and hemicellulose, could be separated, the intrinsic value of lignocellulose as animal feed or as row materials for the production of protein, chemicals and fuels could be better exploited.

To this end, molecular genetics is the preferential tool to characterize the events surrounding the onset of ligninolytic activity, providing a route to identify the relevant genes and analyze the regulation of their expression.

1.1 Plant cell wall structure and composition

New plant cell walls are formed at cell division in the form of the cell plate. After cell plate formation is complete, the cell begins to expand and further wall material is laid down on the cell plate leading to the formation of primary cell wall. Primary cell walls are defined as those that are deposited while the cells are expanding . The middle lamella, which forms a common boundary layer between adjacent cells, occupies the site of the cell plate. Some cell

types usually have only a primary wall at maturity, examples of these are parenchyma and collenchima cells, which are very common cell types in forage plants. In other cell types, sclerenchima fibers and xylem tracheary, wall material is laid down over the primary wall after cell expansion has stopped. This by definition is the secondary wall (Fig. 1).

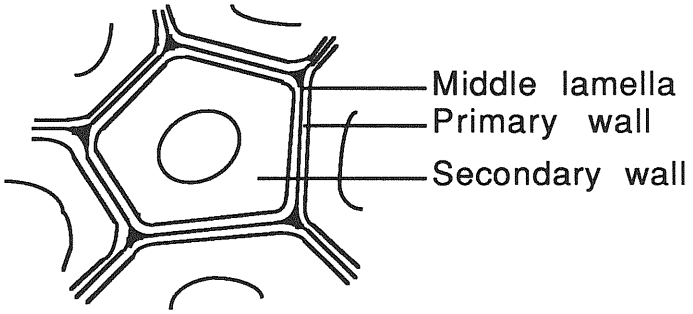


Fig. 1. Wall structure of sclerenchima cells in transverse section.

The wall of higher plants are organized with a cellulosic, fibrillar phase embedded in a matrix phase composed of non-cellulosic polysaccharides, some proteins and lignin. Other wall components include suberin, cutin, tannins, waxes and covalently bound phenolic acids and minerals (Bacic *et al.*, 1988).

1.2 Lignocellulose definition

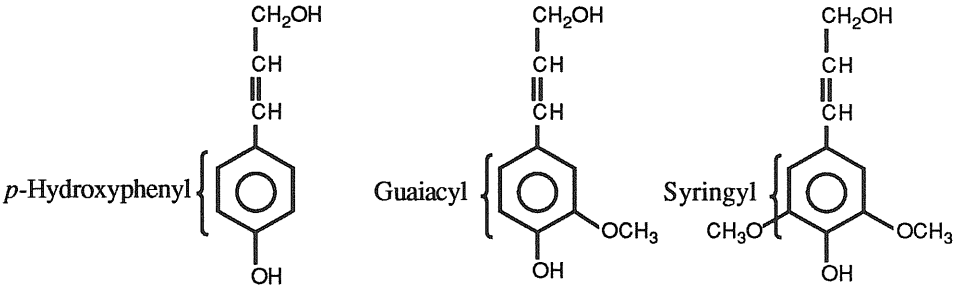
Lignocellulose is a collective term for the three major components of plant cell wall: cellulose, hemicellulose and lignin. Lignocellulose may constitute as much as 89 to 98% of the dry weight of wood (Crawford, 1981). The holocellulose fraction, which includes both cellulose and hemicellulose, is the largest component (63 to 78%), while estimates of 15 to 38% lignin have been made for various hardwood and softwood trees (Sarkanen and Ludwig, 1971).

- Cellulose is located in the primary and secondary cell walls of plant. It is an unbranched polymer of several thousand D-glucose units joined via ß-1,4-glycosidic linkages and is insoluble in water;

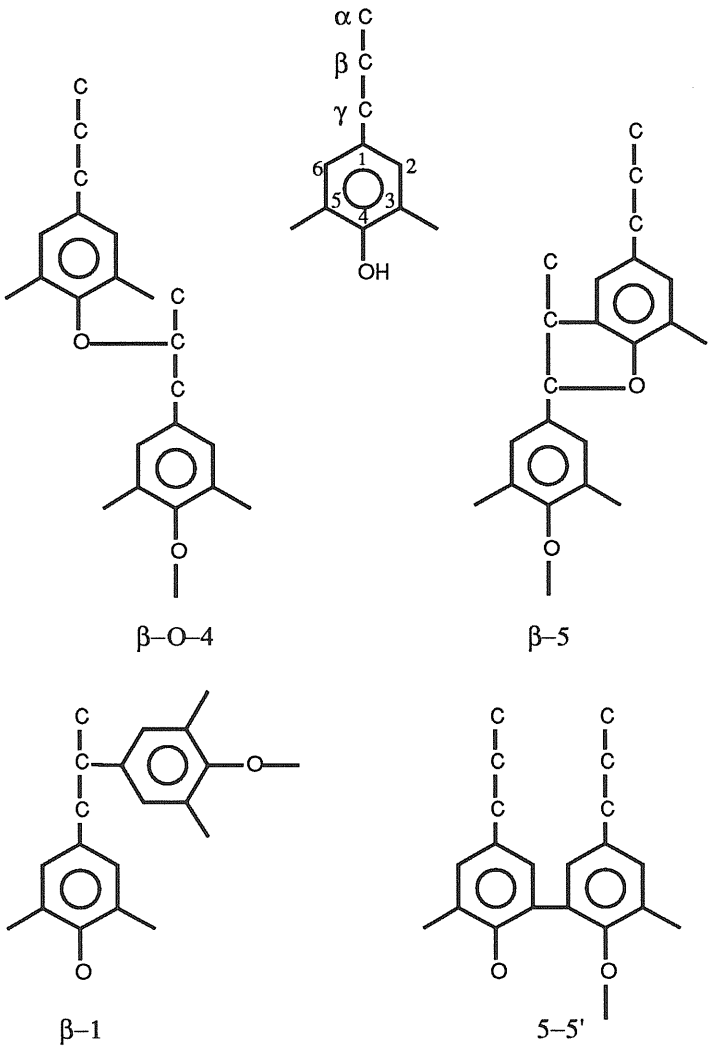
- Hemicellulose is a rather ill-defined group of other structural polysaccharides in plants. They are heteroxylans (glucurono-arabinoxylans), which have a linear (1,4)-linked ß-D-xylopyranosyl backbone substituted by α-L-arabinofuranosyl and α-D-glucuronosyl residues, and smaller amounts of heteroglucans (xyloglucans) and ß-glucans;

- Lignin is a three-dimensional phenylpropanoid polymer synthesized in nature starting from the cinnamyl alcohols, *p*-coumaryl, coniferyl and sinapyl alcohols (Fig. 2a). In lignin these monomers are referred to as *p*-hydroxyphenyl, guaiacyl and syringyl residues and are linked together by different types of bonds, including alkyl-aryl, alkyl-alkyl and aryl-aryl ether bonds (Kirk *et al.*, 1980) (Fig. 2b). Their hydroxyl group is oxidized by peroxidases yielding free radical species that couple in a non enzymatic and random fashion to form dilignols, oligomeric intermediates and finally lignin macromolecules. Lignins from most gymnosperms contain virtually only guaiacyl residues, angiosperms are rich in syringyl nuclei, while lignins from grasses have, in addition to guaiacyl and syringyl residues, significant amounts of *p*-hydroxyphenyl residues.

A question concerning the structure of the lignocellulose is whether or not lignin and holocellulose are covalently bound. Although lignin physically surrounds, but is probably not covalently linked to cellulose, available data suggest the existence of some types of chemical bonds between other plant polysaccharides and lignin (Conchie *et al.*, 1987; Morrison, 1987). There have been many attempts to establish the identity of covalent linkages between polysaccharides and lignin and at present the evidence for such linkages is strong but indirect. Monosaccharides, oligosaccharides or polysaccharides may be bound to phenolic hydroxyls or side chain hydroxyls on lignins by direct glycosidic linkages. Moreover, direct ether cross-linkages between lignin monomers and polysaccharide hydroxyls, or esterification of lignin hydroxyls by carboxyls from uronic acids in wall polymers (eg. glucuronoxylans, rhamnogalacturons) have been postulated. Finally the presence of hydroxycinnamic acids esterified to wall polysaccharides and the detection of



(a)



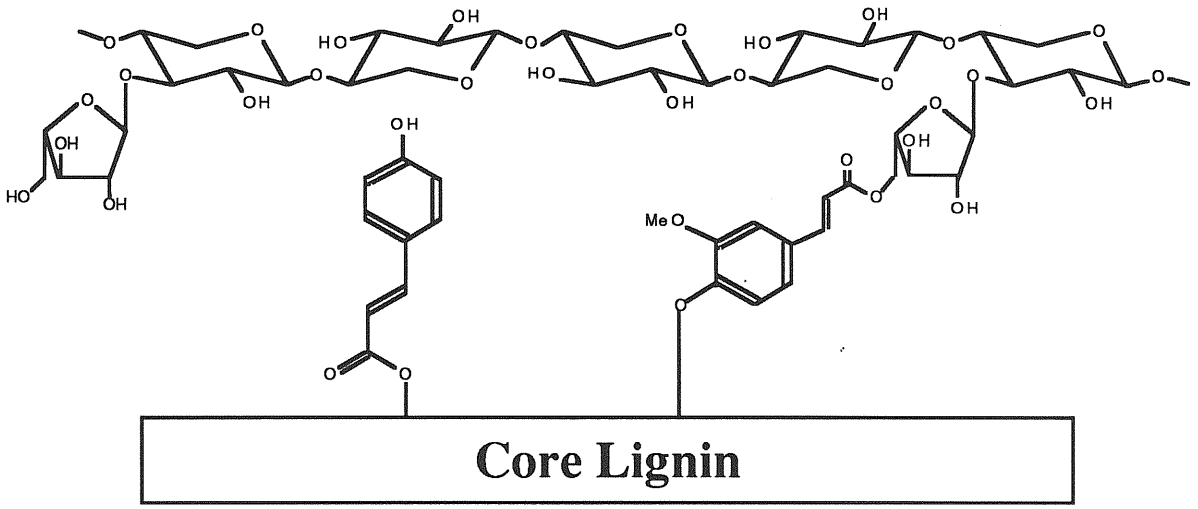
(b)

Fig. 2. (a) Structure of the major alcohol precursors of lignin with the equivalent phenolic acids shown in parenthesis, and **(b)** the four major aryl ether and carbon to carbon linkages found after polymerization of lignin precursors; dimers are shown in skeletal form only.

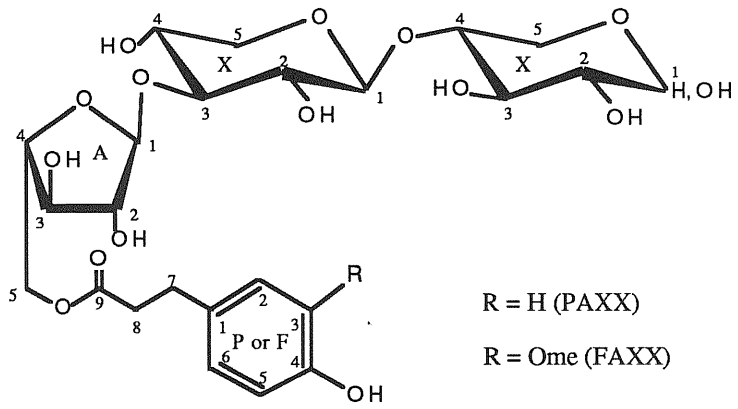
ether linked hydroxycinnamic acids on lignin has led to the proposal that ester linked hydroxycinnamic acids on polysaccharides could also be ether linked to lignin and so form a bridge between the two polymers.

1.3 Core and non-core lignin

The phenolic components of plant cell walls include polymers (core lignin) and low molecular weight monomers (non-core lignin) (Fig. 3a). Both of them have an inhibitory effect on the utilization of the carbohydrate portion of the wall and influence ruminant digestion of cell wall polysaccharides, but also other processes such as biomass conversion and pulping (Hatfield, 1990). The association of non-core lignin phenolic acids, notably *p*-coumaric and ferulic acid, with cell wall polysaccharides has been extensively investigated in plants from several families, including the economically important Gramineae. They are thought to be present as esters because of their release from cell walls on treatment with alkali (Hartley, 1987). Their linkage to cell wall polysaccharides has been studied by determining the structure of water soluble compounds released by treatment of sugar-cane bagasse, wheat-bran and maize cell-walls with "cellulase" (a mixture of polysaccharide hydrolases). The released oligomers, O-[5-O-(*trans*-*p*-coumaroyl)-α-L-arabinofuranosyl]-(1→3)-O-ß-D-xylopyranosyl-(1→4)-D-xylopyranose (PAXX) and O-[5-O-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1→3)-O-ß-D-xylopyranosyl-(1→4)-D-xylopyranose (FAXX), contained *p*-coumaric or *trans*-ferulic acid ester-linked to arabinose which in turn was linked to xylose (Fig. 3b). One in every 31 arabinose residues was esterified with *p*-coumaric acid and 1 in every 15 with ferulic acid. The quantities of PAXX and FAXX released from cell walls of barley straw by treatment with "cellulase" accounted for one sixth of the *p*-coumaric acid and half of the ferulic acid released by treatment with sodium hydroxide (Smith and Hartley, 1983; Mueller-Harvey and Hartley, 1986). These phenolics bound to hemicellulose may indicate the first step toward the synthesis of the lignin polymer and the linkage between lignin and hemicellulose.



(a)



(b)

Fig 3. (a) Schematic structure of the cross linkage of hemicellulose to core lignin via an ester-ether ferulic acid linked moiety; **(b)** PAXX and FAXX chemical structure.

1.4 Microbial degradation of polymeric lignin and low molecular weight lignin-derived molecules

Lacking knowledge of the lignin structure resulted in slow progress in the understanding of lignin biodegradation before 1960's. Since lignin degrading microorganisms apparently can not grow on lignin as the sole carbon source, classical microbial enrichment and isolation method could not be applied. Furthermore, a reliable assay to screen for lignin biodegradation was developed only in the 1970's, when ^{14}C-labeled synthetic lignins (DHP) became available (Kirk *et al.*, 1975; Hackett *et al.*, 1977). Analysis of the soil microflora responsible for aerobic ligninolytic activity by use of specific procaryotic and eucaryotic antibiotic additions to soil in ^{14}C-lignin biodegradation experiments suggested the participation of both bacteria and fungi in lignin mineralization (Zeikus, 1980). However, the most rapid and extensive lignin degraders are certain fungi. Depending on the mode of decay, these wood degrading fungi are classified into three groups, white-rot, soft-rot and brown-rot fungi. Ascomycetes, basidiomycetes and fungi imperfecti have been reported to degrade lignin. Brown-rot fungi have a very limited activity to degrade lignin in wood. They mainly hydrolyze the wood polysaccharides resulting in brown color. These fungi rather humify than degrade lignin (Kirk, 1971). The extent of lignin degradation by soft-rot fungi is low, since their attack is concentrated on wood polysaccharides, while the lignin molecule is only partially degraded. Both brown-rot and soft-rot fungi cause less than 20% $^{14}CO_2$ from ^{14}C-lignin (Haider and Trojanowskj, 1980). In comparison white-rot fungi are able to release up to 70% $^{14}CO_2$ from ^{14}C-lignin under optimal conditions (Hatakka and Uusi, 1983).

The basidiomycete *Phanerochaete chrysosporium* is the most studied and best characterized white-rot fungus (Schoemaker and Leisola, 1990). It penetrates the lumen of wood cells and causes a breakdown of the different components by secreting a whole set of enzymes. It synthesizes two classes of extracellular lignin-degrading peroxidases, ligninases (LiP) (Glenn *et al.*, 1983; Tien and Kirk, 1983) and manganese-peroxidases (MnP) (Glenn and Gold, 1985). The significance of these peroxidase in lignin biodegradation is well

documented. Mutants with lowered peroxidase activity exhibit lowered total ligninolytic activity (Gold *et al.*, 1982). To summarize, it is widely accepted that lignin peroxidases act on non phenolic aromatic substrates oxidizing them by one-electron abstraction to radical cations, which then undergo several non enzymatic reactions leading to the breakdown of lignin and lignin model compounds. Therefore, other enzymes, like Mn(II)-dependent peroxidase and laccase are needed to attack phenolic compounds. Several enzymes, other than LiP and MnP, are involved in the process of lignin depolymerization and degradation by white-rot fungi and have been identify and characterized (Table 1).

Table 1. Enzymes of the ligninolytic system of lignin-degrading white-rot fungi.

Enzyme	Reactions catalyzed	Source
Lignin peroxidase (LiP)	Oxidation of benzylic alcohols Cleavage of C-C bonds Cleavage of C-O bonds Aromatic ring opening Polymerization of phenols	*P. chrysosporium* and most other white-rot fungi .
Mn(II)-dependent peroxidase (MnP)	Oxidation of phenols Cleavage of C-C bonds Cleavage of C-O bonds	*P. chrysosporium* and other white-rot fungi
Laccase	Similar to MnP	*C. versicolor* and other white-rot fungi
Dioxygenase	Aromatic ring cleavage	*P. chrysosporium* *P. ostreatus*
Aryl-alcohol dehydrogenase	Reduction of C α-aldehydes In symphony with LiP (?)	*P. chrysosporium* *Ph. radiata*
NAD(P)H:quinone oxidoreductase	Reduction of quinones	*P. chrysosporium*
Cellobiose:quinone oxidoreductase	Reduction of quinones Cellobiose degradation	*P. chrysosporium*
Aromatic acid reductase	Reduction of aromatic rings	*P. chrysosporium* *Ph. radiata*

Vanillate hydroxylase	Oxidative decarboxylation of vanillic acid	*P. chrysosporium*
Aryl-alcohol oxidase	Oxidation of aromatic C α-alcohols Production of H_2O_2	*Pl. sajor-caju*
Glucose oxidase	Production of H_2O_2	*P. chrysosporium*
Glyoxal oxidase	Production of H_2O_2	*P. chrysosporium*
Methanol oxidase	Production of H_2O_2	*P. chrysosporium*

Bacterial counterparts of fungal ligninases have not yet been found, however bacteria isolated from habitats where lignocellulose material is degraded, such as soils containing lignified decaying plant material or from anaerobic ecosystems in sediments and in the rumen, can be expected to metabolize lignin or lignin-derived compounds. Several ultrastructural studies have shown that bacteria can erode wood fibers through both tunnelling and cavitation (Daniel *et al.*, 1987; Singh *et al.*, 1988). *Actinomycetes* degrade grass tissues producing water soluble residues, called "acid precipitable polymeric lignin"(APPL) (Crawford *et al.*, 1983; Pettey and Crawford, 1985), that contain polysaccharides and lignin, which is resistant to further oxidation to CO_2. Bacterial activity on polymeric lignin substrate is limited and low rates of conversion of radiolabelled lignin substrates to $^{14}CO_2$ are observed with most of the bacteria tested, on the contrary bacteria of several genera, including *Pseudomonas*, *Alcaligenes*, *Arthrobacter*, *Nocardia* and *Streptomyces* readily degrade low molecular weight compounds structurally related to lignin, that build up the lignin polymer itself (Vicuña, 1988; Vicuña *et al.*, 1993).

Much of the present knowledge of the mechanism of lignin degradation by bacteria has been obtained using as substrates lignin model compounds (LMC), which have already been of great importance in the discovery of ligninases from *Phanerochaete chrysosporium.* These compounds, usually dimeric, contain typical substructures and linkages which occur in the lignin polymer. Phenylcoumarans, cyclic lignans, biphenyls, and compounds with ß-O-4 and ß-1 linkages are the most often used LMC. Although studies on bacterial degradation of lignin-related structures other than single ring aromatic compounds are

scanty, they have already provided valuable information. There is evidence that various strains can indeed grow on oligolignols, this implies that these microorganisms produce enzymes that catalyse cleavage of interunit bonds (Jakela *et al.*, 1987; Samejima *et al.*, 1987), however these bacterial enzymes seem to be more specific than fungal ligninases. This can be deduced from the observation that a particular strain may grow on a ß-O-4 model, but not on a ß-1 model and *vice-versa.* (Vicuña *et al.*, 1987). In contrast, lignin peroxidases catalyses rupture of ß-O-4 as well as ß-1 models among others. Consequently, complete depolymerization of the macromolecule by a particular bacterial strain would require the simultaneous presence of several types of enzymes. In addition, these enzymes are not secreted, therefore lignin-related dimeric and even tetrameric compounds are metabolized presumably within the cell, which would explain how the size of lignin-polymer could be another factor limiting its degradation by bacteria (Kirk and Farrell, 1987). Besides simplifying the study of the enzyme-catalysed breakage of the different types of linkages in the lignin macromolecule, the model compounds are also suitable for studying other reactions involved in lignin breakdown, such as side chain modification, ring fission and demethoxylation.

Most natural monomeric intermediates in lignin catabolism are methoxylated and the removal of the methyl (or other alkyl) group is an essential step in preparing the molecule for ring cleavage(Ander *et al.*, 1987). The Ar-OCH_3 ethers are resistant to hydrolytic attack and bacteria, as well as fungi, attack them oxidatively by a mono-oxygenase to generate the corresponding phenol. After demethylation there are three other stages in the formation of the dihydroxyphenols susceptible to ring fission: (1) oxidation of the C_3 side chain in phenylpropanoids arising from the polymeric or dilignol precursors, (2) the oxidation of the alcohol and aldehyde groups at C-1 of the ring to the carboxyl group and (3) hydroxylation of monophenols to appropriate diphenols. Finally ring fission of the 1,2-dihydric phenols occurs by intradiol (ortho) or extradiol (meta) cleavage. Ortho fission involves the oxidative rupture of a bond between adjacent carbon atoms bearing the hydroxyl groups, while meta fission involves the oxidative rupture of the bond between a carbon atom bearing a hydroxyl

group and one bearing a hydrogen atom or side chain substituent. The subsequent stages in the degradation of the ring fission products of the ortho and meta mechanisms are biochemically distinct and their end products are different (Cain, 1980).

Catabolism of lignin related aromatic compounds is not a strictly aerobic process. In 1934 Tarvin and Buswell provided the first evidence that the aromatic nucleus of common aromatic compounds was completely decomposed when incubated anaerobically with sewage sludge. Anoxic ecosystems are created when oxygen consumption exceeds its supply, eg. in soils with impeded drainage, stagnant water, sewage treatment digester, the alimentary tract of all animals, sediment of oceans and industrial plants. In the absence of molecular oxygen the metabolic fate of aromatic compounds and their mineralization to CO_2 (and CH_4) depends on the availability of light or inorganic electron acceptors such as NO_3^-, SO_4^{2-} or CO_2. In fact, anaerobic metabolism of aromatic monomers in absence of molecular oxygen (reviewed by Colberg, 1988 and Evans, 1988) is known to occur during anaerobic photometabolism, under nitrate reducing conditions, under sulphate-reducing conditions, in microbial consortia where fermentation is coupled with methanogenesis and by pure cultures of fermentative bacteria. In all these cases the biochemical feature that unites the anaerobic catabolism of diverse aromatic compounds is the involvement of a common reductive pathway for ring cleavage. The aromatic rings are first reduced by hydrogenation reactions and then ring cleavage occurs by hydration. Demethoxylation, decarboxylation and other reactions which remove aromatic ring substituent groups, are all possible under strictly anaerobic conditions. There are microorganisms which remove only the substituent groups leaving the aromatic ring intact. However, in the presence of other microorganisms they contribute to the cleavage of the aromatic ring. Ferulic acid, for example, is biodegraded to methane and carbon dioxide by a consortium of bacteria under strictly anaerobic conditions (Healy *et al.*, 1980; Grbic-Galic and Young, 1985). Pure cultures of facultative anaerobes are capable of degrading substituted aromatic acids to aliphatic products under strictly anaerobic conditions (Grbic-Galic, 1986). Strictly anaerobic bacteria have also been described to be able to cleave rings of aromatic monomers. Phloroglucinol, for example, is

fermented by two anaerobic rumen isolates, *Coprococcus* spp. and *Streptococcus bovis* (Tsai and Jones, 1975; Patel *et al.*, 1981).

Degradation studies in strictly anaerobic conditions using ^{14}C-oligolignols, synthetic or derived from lignin, and sewage sludge or lake sediment as inoculum indicated that anaerobic microorganism are able to cleave intermonomeric bonds (Colberg and Young, 1982; Zeikus *et al.*, 1982) and release lignin monomers which are known to be degraded to CO_2 and CH_4 under strictly anaerobic conditions.

1.5 Microbes and lignocellulose degradation in the rumen

The potential degradation of lignocellulose by ruminants has been investigated by microbiologists and nutritionists. The first section of the ruminant gastro-intestinal tract, the rumen, is a fermentation chamber filled with microorganisms that are largely responsible for feed digestion. The rumen microbes are not only the agents producing the forage-digesting enzymes and the fermentation acids oxidized by the host, they themselves constitute the host's supply of proteins and other essential nutrients. Rumen microorganisms are found in suspension, in association with the solid digesta and adherent to the rumen wall, and properties of these three populations might be different. In the functional rumen, in which the distribution of the feed particles is affected by contraction and relaxation of the rumen wall, there is a dynamic equilibrium among these populations, because bacteria enter the fluid from the rumen wall and from the particle-associated population and *vice versa* from the fluid they attach to the wall and particles. The major portion of the rumen microbial population consists of strictly anaerobic bacteria and ciliate protozoa, which appear to account for most of the fermentative activity in this organ. Smaller numbers of facultatively anaerobic bacteria, aerobic bacteria, flagellate protozoa, fungi and mycoplasma are also present.

Microbial numbers and the composition of the population are affected by a number of factors of which diet is one of the most important. The very large number of bacteria present

in the rumen (up to 10^{11} viable cells/ml) have been recognized (Hungate, 1966; Hobson, 1988). The rumen contains representatives of all the major morphological forms of small bacteria, with gram-positive and gram-negative rods, cocci, vibrios and helices occurring singly, in chains and in clumps. The rumen is an open system; although certain organisms are repeatedly found to be present and clearly occupy a secure ecological niche, many of the bacteria capable of growth in the rumen are not generally regarded as true rumen bacteria, since they have been isolated only on relatively few occasions under specialized conditions. Aerobic organisms isolated from ruminants are *Acinetobacter* spp., *Pseudomonas aeruginosa*, *Alkaligenes faecalis*, *Micrococcus varians* and *Flavobacterium* spp. Of the facultative anaerobes various staphylococci and streptococci can be found in the rumen. Coliforms are present in large numbers in the flora of lambs and calves , but their number decline in mature animals. *Bacillus licheniformis*, *B. circulans*, *B. coagulans*, *B. laterosporus* and isolates resembling *B. pumilus* have been detected in the rumen ecosystem (Williams and Withers, 1983). Rumen bacteria species isolated frequently are the anaerobes *Bacteroides* spp., *Streptococcus bovis*, *Ruminococcus* spp., *Butyrivibrio fibrisolvens*, *Wolinella succinogenes*, *Veilonella parvula*, *Eubacterium* spp., *Clostridium* spp. and several rumen methanogens. In addition to bacteria many protozoa are present. Yeast and aerobic fungi are considered transient and non functional, entering the rumen with the feed, while strictly anaerobic fungi have been identified, but only one genus *Neocallimastix* has been described as a legitimate rumen inhabitant.

Cellulose and hemicellulose are potentially totally digestible in the rumen by microbes. Lignin was initially considered "indigestible" in ruminants, however experimental data have shown that lignin is digested to a variable extent with values ranging from 2% to 53% (Susmel and Stefanon, 1993). A unidentified, facultatively anaerobic bacterium isolated from rumen fluid that preferentially attacks lignin-containing cell walls of Bermuda grass and ferments several lignin-derived monomers including ferulic acid, sinapinic acid and *p*-coumaric acid, has been described (Akin, 1980). Microbial consortia from rumen fluid

degrade different lignocellulosic substrates and lignin monomers in vitro (Chen *et al.*, 1985; Akin and Rigsby, 1987; Akin and Benner, 1988; Borneman *et al.*, 1989).

Forages, silages or by-products containing large amounts of lignocellulose usually make up a significant portion of ruminants' diets. Digestibility of feed nutrients determines the animal's productive efficiency and it is well known that lignin reduces the degradation of plant material by rumen microbes. Lignin is the major cell wall constituent leading to decreased structural carbohydrate utilization, but the chemical basis for the suppression in digestibility has not been elucidated. Complexes of plant carbohydrates with polymeric lignin or phenolic monomers have been shown to inhibit the availability of the carbohydrates for digestibility (Jung and Ralph, 1990) and the limitation of growth on *p*-coumaric and ferulic-carbohydrate complexes (FAXX and PAXX) varied with individual bacteria and appeared to be influenced by the ability to hydrolyze carbohydrate linkages (Akin *et al.*, 1993). In addition to the binding of structural carbohydrates by phenolic compounds, free phenolic compounds have been shown to inhibit the growth or activity of rumen bacteria, protozoa and fungi *in vitro* (Chesson *et al.*, 1982; Akin, 1988). When phenolics are progressively removed from graminaceous cell walls by alkali treatment, there is a gradual increase in the biodegradability of the wall polysaccharide and *p*-coumaric and ferulic acid are the main phenolic monomers that are released from graminaceous cell walls by saponification. Consequently it has been postulated from experimental data that soluble phenolics, particularly *p*-coumaric and ferulic acids, free or as carbohydrate esters, inhibit ruminal fiber fermentation by disruption of bacterial metabolism, whereas phenolics/lignin bound to the cell wall restrict access to polysaccharide for enzymatic hydrolysis.
Such a situation implies that digestibility could be improved by reducing the lignin content of a feedstuff or perhaps by removing its inhibition of ruminal digestion.

1.6 Engineering rumen flora

Digestion of feedstuffs is the limiting factor of animal production, and for the ruminant this process is determined by the efficiency of microbial activities in the rumen. Therefore, animal production may be improved by i) enhancing rumen microbial cellulase, hemicellulase, amylase and lignin esterase activities to increase the amount of energy transferable to animal, ii) modifying microbial fermentation patterns or, iii) optimizing microbial growth. In addition, novel metabolic activities can be introduced in the rumen and microbial detrimental activities, such as conversion of initially harmless compounds to harmful products, can be inhibited. Traditionally, increases in efficiency of rumen function have been obtained by altering the feedstock and by using antibiotics to alter the predominant microbial species. An alternative approach would be to manipulate rumen function through genetic engineering. Genetic engineering technology is being developed for rumen bacteria: stable vectors have been constructed and efficient transfer processes are now available. Shuttle vectors and naturally occurring self transmissible plasmids have been developed for *Bacteroides ruminicola* and *Butyrivibrio fibrisolvens*. Streptococcal transposons have been introduced into *B. fibrisolvens*; and *Streptoccocus bovis*. Phage systems were also reported to be undergoing development for *S. bovis* (Wallace, 1992). Recently the rumen bacterium *Butyrivibrio fibrisolvens* has successfully been genetically modified to detoxify fluoroacetate, a poisonous component of trees and shrubs in Australia (Gregg, 1995). Therefore, it remains to be shown experimentally whether the detoxification that has been observed *in vitro* will be adequate *in vivo*. This final step, the introduction of a genetically engineered microorganism into the rumen is the most difficult obstacle to overcome. Two major constraints for the establishment of a microbial species in the rumen are the short turnover time in the rumen and competition with other microorganisms. If rumen fermentation has to be significantly altered by the new microorganism it has to become predominant in the rumen and it needs a competitive advantage. This can be done by altering the ecosystem adding antibiotics or novel substrates. Alternatively, degradation of low levels of toxic compounds, production of antibiotics to control other microorganisms or production

of compounds that act directly on the animal would not require that a genetically engineered microorganism becomes a predominant species, but requires strategies for maintaining it in the rumen for the length of time appropriate for each production system in a minor proportion. Rumen components to which a genetically engineered microorganism could become attached include (a) highly lignified portions of biomass, which are degraded slowly, (b) other microorganisms such as protozoa, which tend to sequester them, (c) the rumen epithelial wall (Patterson, 1989).

There can be little doubt of the economic gains that could result for applying genetic manipulation of rumen bacteria to improve capability of animals and reduce effects of toxic and antinutritive compounds. In practical terms the technology is developing, but there are questions of how well the modified organism would spread and persist in a ruminal population under field conditions. Most importantly, the possible environmental effects of releasing genetically modified organisms must be examined.

2. MATERIALS AND METHODS

2.1 Organisms and plasmids

2.1.1 Bacteria

B. pumilus PS213 was isolated from cow rumen fluid in our laboratory.

B. licheniformis LMG 6933, *B. circulans* LMG 6926, *B. laterosporus* LMG 6931, *B. macerans* 6324 were obtained from the Culture Collection of Laboratorium voor Microbiologie, Universiteit Gent.

B. subtilis 1012 (S. Bron, Biologish Centrum, Haren).

E. coli DH5α (*endA*1, *hsdR*17, *supE*44, *thi*-1, *recA*1, *gyrA*96, *relA*1, Δ*lac*U169 (*ϕ*80*lacZ*ΔM15))(Hanahan, 1983).

Stocks of *E. coli*, untransformed or containing plasmids, and *Bacillus* spp. were stored at -80°C in 30% glycerol and 70% nutrient Luria-Bertani (LB) medium. For culture of cells containing plasmid, an appropriate antibiotic was present in the medium.

2.1.2 Yeasts

Saccharomyces cerevisiae YPH250 ([cir^0] a *ura*3-52, *lys*2-801, *ade*2-101, *his*3-Δ200, *leu*2-Δ1, *PAD1::URA3*).

2.1.3 Plasmids

The 2,686 bp *E. coli* plasmid pUC19 was used. It contains parts of pBR322 and M13mp19 (Yanisch *et al.*, 1985). It has a high copy number and carries a 54 bp multiple cloning site (MCS) polylinker that contains sites for 13 different restriction enzymes.

YCp50, a 8,127 bp yeast centromeric plasmid, was used to study the expression of the ferulic acid decarboxylase gene in yeast. It contains a 1.75 Kb *Pvu*II-*Eco*RI fragment with the *CEN*4 element and a 0.8 Kb *Eco*RI-*Hin*dIII fragment with the *ARS*1 element, inserted

into the *Pvu*II site of YIp5 (Struhl *et al.*, 1979). The plasmid can replicate in *E. coli* and carries the genes for tetracycline and ampicillin resistance from pBR322 to use as selectable markers in *E. coli*. The *URA*3 gene from *S. cerevisiae* is present to use as a selectable marker in yeast strains defective for this gene.

Another plasmid used for this purpose was the 6,875 bp yeast expression vector pVT100-U (Vernet *et al.*, 1987). It is an yeast/*E. coli* shuttle vector carrying the *S. cerevisiae* 2μ ori. Genes can be expressed in yeast by insertion within a MCS located downstream the *ADH*1 promoter.

2.2 Media

Luria-Bertani (LB) Medium

1%	Tryptone
1%	NaCl
0.5%	Yeast Extract

pH 7.0

Where solid medium was required, agar was added to 1.5%. Medium was sterilized by autoclaving (120°, 1.0 bar pressure for 20 minutes).

When needed, ampicillin and kanamycin were added to 100 μg/ml from a filter sterilized 50 mg/ml stock solution in redistilled water. For selecting *B. subtilis* mutants 5 mg/ml of kanamycin were used. Tetracyclin was added to 12.5 μg/ml from a 5 mg/ml solution in absolute ethanol. At times, 100 μl of the chromogenic substrate X-gal was overlayed onto solid medium, from a 20 mg/ml stock in N-N-dimethylformamide for the detection of colonies derived from cells expressing the enzyme ß-galactosidase. Moreover, 50 μl of 1M IPTG in water were added to the same plates.

M9CA

0.6%	Na_2HPO_4
0.2%	KH_2PO_4
0.05%	NaCl
0.1%	NH_4Cl
0.2%	Casamino acid

pH 7.4

After being autoclaved, 2 ml of filtered $MgSO_4$ 1M and 0.1 ml of filtered $CaCl_2$ 1M were added to 1 litre.

Fluid Thioglycollate medium (Difco)

1.5%	Casitone
0.5%	Yeast extract
0.55%	Dextrose
0.25%	Sodium chloride
0.05%	L-Cysteine
0.05%	Sodium thioglycollate
0.075%	Agar
0.0001%	Resazurin

pH 7.0

Medium was CO_2-bubbled, sterilized and dispensed into test tubes in anaerobic atmosphere.

Soybean meal-glucose medium

2%	glucose
0.5%	Yeast extract
0.5%	Soybean
0.5%	NaCl
0.5%	K_2HPO_4

pH 7.0

The medium was autoclaved after having adjusted the pH.

YPD

1% Yeast extract

1% Peptone

1% Dextrose

Sterilized by autoclaving

2.3 Lignin related aromatic compounds

The lignin related aromatic compounds were purchased from either Sigma, (Sigma Chemical Company, St. Louis, Missouri) or Aldrich (Aldrich srl, Milwaukee, Wisconsin) as the following: ferulic acid, syringic acid, syringaldehyde, 3,4-dimethoxy-benzaldehyde (veratryl aldehyde), 3,4-dimethoxybenzyl acid (veratric acid), 3,5-dimethoxy-4-hydroxycinnamic (sinapic acid), benzoic acid, *p*-hydroxybenzoic acid, protocatechuic acid, *p*-coumaric acid, *trans*-cinnamic acid, vanillin, vanillic acid. Vinylguaiacol was purchased from Lancaster (Lancaster Synthesis GmbH, Mühlheim am Main, Germany).
All lignin related compounds were prepared as 5 g/l stock solutions in water, filter sterilized and added to the autoclaved media.

2.4. Culture methods

2.4.1 Test for the utilization of phenolic compounds *in vivo*

To check the ability of a bacterial or yeast strain to utilize a monomeric aromatic compound, the strain was inoculated into LB or Thioglicollate or Soybean meal-glucose medium containing 0.5 g/l of the interesting compound. After an interval of growth an aliquot of the culture was collected and centrifuged. The supernatant was diluted 20 times in 0.2 M Tris

pH 7.2 and analysed by UV spectrophotometer. The UV spectrum (between 200 and 400 nm) was compared to the spectrum of the substrate in the same medium before the inoculum. Alternatively an HPLC analysis was performed.

2.4.2 Test of inducibility of the ferulate decarboxylase activity in *Bacillus* spp.

100 µl of a starter culture were inoculated into 20 ml of LB and 20 ml of LB plus 0.5 g/l ferulic acid respectively and incubated overnight at 37°C. The following day each culture was centrifuged and the pellet resuspended to the same optical density in fresh LB medium plus 0.5 g/l ferulic acid. The utilization of ferulic acid was monitored at different intervals by spectrophotometer analysis comparing the spectra from the induced and non induced culture.

2.5 DNA techniques

2.5.1 Large scale chromosomal DNA purification

Total DNA extraction and chromosomal DNA purification were carried out as a modification of the protocol described by Ausubel *et al.*, 1988. Cells were grown overnight at 37°C in a 2 liter flask containing 500 ml of LB medium. Culture was sedimented in a Sorvall RC-5B centrifuge (Du Pont, Wilmington, Delaware) in 250 ml bottles using a GSA rotor at 4,000Xg at 4°C for 10 minutes. Supernatant was discarded and the pellet was resuspended in 23.75 ml of TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0). Lysis was obtained with 600 µg/ml of lysozyme and 15 minutes of incubation at 37°C. After incubation 1.25 ml of 10% SDS and 100 µl of 20 mg/ml proteinase K were added, mixed thoroughly and left 1 hour at 37°C. The mixture was transferred to 40 ml Oak Ridge tubes with 4.5 ml of 5 M NaCl and mixed. Then 4.25 ml of CTAB/NaCl solution were added and incubated 20 minutes at 65°C. Extraction was made with an equal volume of chloroform/isoamyl alcohol and the two phases were separated by spinning at 6,000Xg in a Sorvall RC-5B (Du Pont) centrifuge using a SS-34 rotor at 4,000Xg at room temperature. The aqueous supernatant

was transferred to a 50 ml tube with a wide-bored pipet. 0.6 volumes of isopropanol were added and the solution mixed until a stringly white DNA pellet precipitated out of the solution and condensed into a tight mass. The precipitate was transferred to 1 ml of 70% ethanol in a fresh 2 ml tube, by hooking it by the end of a Pasteur pipet that was bent and sealed in a Bunsen flame. Pellet was spun 5 minutes at 14,000 rpm in an Eppendorf Centrifuge 5415 C (Eppendorf-Nether-Hinz GmbH, Hamburg, Germany). Supernatant was discarded and the pellet was dried under vacuum and then it was resuspended in 4 ml of TE. DNA concentration was measured at the spectrophotometer. A 12.5 ml CsCl gradient was prepared transferring a volume of solution containing about 1 mg of DNA into a 50 ml tube and adjusting the weight to 10.4 using TE. 11.3 g CsCl were added and the salt was dissolved before adding 1.3 ml of 2 mg/ml ethidium bromide. It was spun 10 minutes at low speed in a Sorvall RT6000B centrifuge (Du Pont) and avoiding the floating debris, the solution was dispensed into a 12.5 ml polyallomer Quick-seal Beckman centrifuge tube (Beckman Instruments, Inc., Fullerton, California). These tubes were spun 8 hours at 54K rpm in Beckman L8-70M Centrifuge using the vertical rotor Vti65. The chromosomal DNA band was visualized under a long wave UV lamp, and recovered by puncturing the tube with a syringe bearing a 18-gauge needle, as described by Sambrook *et al.*, 1989. Ethidium bromide was removed by extraction with water-saturated *n*-butanol. One volume of n-butanol was added to the recovered DNA solution, thoroughly mixed, centrifuged at 2,000 rpm in a Sorvall RT6000B centrifuge for 3 minutes at room temperature. The lower aqueous phase was recovered and the extraction repeated until the solution would become clear. CsCl was removed by dialysis for 48 hours against several changes of 1 liter TE (pH 8.0) at 4°C in dialysis bags. DNA concentration and purity were calculated spectrophotometrically by reading the absorbance at 260 and 280 nm, and assuming that 1 OD_{260} = 50 µg/ml of double strand DNA. Purity of the sample was determined by the ratio $O.D._{260}/O.D._{280}$, considering a value of 1.5 or higher as a pure DNA preparation (Sambrook *et al.*, 1989). DNA was stored at 4°C until needed.

2.5.2 Large scale preparation of plasmid DNA

Plasmid containing cells were grown overnight at 37°C with shaking in a 2 liter flask containing 500 ml LB medium supplemented with 100 μg/ml ampicillin. Cells were harvested by centrifugation in a Sorvall RC-5B (Du Pont) centrifuge in 250 ml bottles using a GSA rotor at 4,000Xg at 4°C for 10 minutes. Pellet was resuspended completely in 10 ml TE containing 100 μg/ml RNAase. Bacterial cells were lysed and cellular proteins, chromosomal and plasmid DNA were denatured by adding 10 ml of 200 mM NaOH and 1% SDS (Birnboim and Doly, 1979). The solution was mixed gently and incubated 5 minutes at room temperature. Precipitation of cellular debris, denatured proteins and high molecular weight nucleic acids from the aqueous phase was achieved by mixing well the solution with 3 M potassium acetate pH 5.5 followed by incubation on ice for 15 minutes. The mixture was pelleted in a Sorvall SS34 rotor at 30,000Xg for 30 minutes at 4°C. After centrifugation the supernatant was loaded onto an equilibrated QIAGEN-tip 500 (Qiagen Inc., Chatsworth, California) and allowed it to enter the resin of the column by gravity flow. Column was washed twice and finally DNA eluted using buffers provided by the Qiagen Kit. DNA was precipitated with 0.7 volumes of isopropanol and centrifuged in a Sorvall RC-5B (Du Pont) centrifuge at 15,000Xg, at 4°C for 30 minutes. Supernatant was carefully removed. The pellet was dried and resuspended in TE.

2.5.3 Bacterial plasmid DNA minipreps

Plasmid DNA was purified with the modified alkaline lysis procedure described by Zhou *et al.*, 1990. A single bacterial colony containing the plasmid of interest was inoculated in 5 ml of liquid LB in the presence of the appropriate antibiotic and incubated at 37°C in a shaker-incubator overnight. Two ml of bacterial cells were collected in a 2 ml Eppendorf tube and centrifuged for 1 min at 14,0000 rpm in an Eppendorf Centrifuge 5415 C. The supernatant was almost completely removed, leaving approximately 100 μl of supernatant in which cells were resuspended by vortexing. 300 μl of TENS solution (TE buffer containing 0.1N NaOH and 0.5% SDS) were added, and the mixture vortexed for a few seconds. After adding 150 μl of 3.0 M sodium acetate, pH 5.2, the mixture was vortexed again and then

spun for 2 minutes to pellet cell debris and chromosomal DNA. The supernatant was transferred into a fresh tube and mixed well with 0.9 ml of 100% ethanol which has been precooled to -20°C. Plasmid DNA was pelleted by 2 minutes of centrifugation as above. The supernatant was discarded, then the pellet was washed with 70% ethanol and dried under vacuum, then was resuspended in 40 ml of TE buffer and stored at 4°C until needed. RNA was degraded during the incubation with restriction endonucleases by the addition to the reaction mixture of 25 µg/ml RNAase.

2.5.4 Restriction endonuclease digestion of DNA

Samples of either purified plasmid DNA or total cellular DNA were typically totally digested in a minimum reaction volume of 15 µl. Reactions were performed according to the enzyme manufacturer specifications. For a typical reaction 2-4 µl of CsCl purified DNA were digested with 2-4 units of enzyme after having added 1.5 µl of 10x buffer and water to 15 µl. The reaction was terminated, after incubation 2 hours at 37°C, by 1) the addition of 1/6 volume of 6X DNA sample buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Fycoll in water) and subsequently run on an agarose gel; 2) the addition of 1/10 volume of sodium acetate 3 M and precipitation with 2.5 volumes of ethanol.

2.5.5 Agarose gel electrophoresis

Gels were prepared and run as described by Sambrook, *et al.*, 1989. DNA samples were usually run on 0.5-0.8 % agarose gel prepared in 0.5X TBE buffer (0.045 M Tris-borate, 0.001 M EDTA pH 8.0), and melted on a hot plate or in a microwave. After the mixture had cooled at less than 60°C, the agarose was poured into a gel mould and let solidify. Once the gel solidified it was placed into the gel apparatus and submerged with 0.5X TBE buffer. DNA samples were loaded on the wells after having added 1/6 volume of 6X DNA sample buffer. The gels were run at approximately 8 V/cm. Gels were stained by immersion in a solution of 0.5 µg/ml of ethidium bromide, and visualized under UV light.

2.5.6 QIAquick purification of DNA fragments

The DNA plasmid or fragment to be purified, could be recovered either from an aqueous solution or from an agarose gel slice with the QIAquick purification kit (Qiagen Inc.). From an aqueous sample, 5 volumes of buffer PB were added to the solution. For TBE gel slices 3 volumes of buffer QX1 were added and gel slice solubilized by heating and occasional mixing, at 50°C. Then the sample was loaded on a QIAquick spin column and centrifuged. The column was washed with 750 µl of buffer PE. Finally, to elute DNA, 50 µl 10 mM Tris/HCl pH 8.5 were added and the column was spun.

2.5.7 DNA ligation reactions

Ligation of DNA fragments to plasmid vector was essentially performed essentially as described by Sambrook *et al.*, 1989. Ligation reactions were incubated at 16°C overnight and were typically performed in the smallest possible volume to increase the frequency of interaction between the different DNA fragments. Additionally, DNA was added to the reaction at a molar insert to vector ratio of at least 3:1. For blunt end ligations this ratio was sometimes increased at 10:1 or greater. The basic reaction mixture was made according to the specifications of the individual supplier of T4 DNA ligase. Following overnight incubation, the reactions were either added directly to competent *E. coli* cells for transformation, or an aliquot was first analyzed by agarose gel electrophoresis to visualize the formation of higher molecular weight ligation products.

2.5.8 Dephoshorylation of linearized plasmid DNA

1-20 µg of linearized plasmid DNA were incubated in 50 µl of supplied reaction mixture with 0.1U of calf intestinal alkaline phosphatase (Pharmacia, Uppsala, Sweden) and incubated 30 minutes at 37°C. Enzyme was inactivated at 85°C for 15 minutes.

2.5.9 Klenow reaction to modify free DNA fragment ends

To generate flush ends on DNA fragments containing 3' recessed termini, the DNA was treated as described by Ausubel *et al.*, 1988. In a 20 µl of digestion reaction, containing

from 0.1 to 4 μg of DNA, 1 μl of 0.5 μM dNTPs (Pharmacia) and 1U of Klenow fragment (Boehringer, Mannheim, Germany) were added. The reaction was incubated at 37°C for 30 minutes. The DNA was purified by Qiaquick kit.

2.5.10 Preparation of competent *E. coli* cells and DNA transformation

E. coli competent cells were prepared according to Sambrook et *al.*, 1989, with some modifications. The recipient strain was inoculated in 5 ml medium in a sterile culture tube and was grown with vigorous shaking at 37°C, overnight, to obtain a stationary phase culture. A 100 μl aliquot of this culture was then transferred aseptically to 25 ml of fresh LB in a 100 ml Erlenmeyer flask. This culture was grown, again with vigorous shaking, at 37°C until reaching an $O.D._{600}$ of 0.4. The cells were placed in an Oak Ridge tube and cooled on ice for 15 minutes. Afterwards, the cells were pelleted at 4,000 rpm in a Sorvall SS34 rotor for 10 minutes at 4°C. The cell pellet was drained well and suspended in 25 ml of ice cold 100 mM $MgCl_2$. Cells were spun as above, pellet was resuspended in 25 ml of 100 mM $CaCl_2$ and left overnight on ice. Competent cells were pelleted again and resuspended in 1.2 ml of 100 mM $CaCl_2$. Aliquots of 0.2 ml of this suspension were frozen in isopropanol-dry ice bath after adding 15% (v/v) glycerol and dispensed at -80°C until needed. Transforming DNA was added to thawed *E. coli* competent cells, and tubes were incubated on ice for 1 hour. Cells were then heat-shocked by incubation in a 42°C water-bath for 2 minutes, after which were grown in 2 ml LB at 37°C for 2 hours. Aliquots of 100, 200, 300 μl were plated on selective medium and grown overnight at 37°C. Transformant colonies were white on X-gal chromogenic substrate or were screened by replica plating on medium containing the second selective marker .

2.5.11 Yeast transformation by electroporation

S. cerevisiae was grown at 30°C in 100 ml of YPD with vigorous shaking to an $O.D._{600}$=1.3-1.5. Cells were harvested by centrifugation at 4,000Xg in a refrigerated Sorvall RT6000B rotor for 5 minutes at 4°C and washed twice with ice-cold sterile water. Then, they were resuspended in 4 ml of ice-cold sterile 1 M sorbitol, centrifuged as above

and finally resuspended to 0.1 ml of ice-cold 1 M sorbitol. In a sterile polypropylene tube 40 µl of the yeast suspension were mixed with about 0.1 µg of plasmid DNA and incubated on ice 5 minutes. This mixture was transferred to a pre-chilled 0.2 cm electroporation cuvette (Bio-Rad, Hercules, California). The cuvette was placed in the chamber of the Gene Pulser apparatus (Bio-Rad, Richmond, California) set to 1.5 kV and 25 µF. The Pulse Controller (Bio-Rad) was set to 200 Ω. After having applied one pulse at the settings defined above, the cuvette was removed from the chamber and immediately 1 ml of cold 1 M sorbitol was added and transferred to a culture tube. Aliquots of the transformation were spread on selective plates containing 1 M sorbitol.

2.5.12 Purification of synthetic oligonucleotides

Oligonucleotides were synthesized by the ICGEB oligonucleotide service, and delivered already deblocked, in solution with ammonium hydroxide. They were purified as described in Sambrook *et al.*, 1989. The oligonucleotide solution was evaporated in a centrifugal evaporator, resuspended in 1 ml of sterile water and centrifuged at 12,000Xg for 5 minutes. The supernatant was transferred to a sterile microfuge tube and extracted three times in succession with 400 µl of 1-butanol discarding the upper organic phase after each extraction. The solution was evaporated again as described above and the pellet was re-dissolved in 500 µl of sodium phosphate buffer pH 6.8. The solution was desalted through a Pharmacia NAP-25^{TM} Column. Concentration was calculated spectrophotometrically by reading the absorbance at 260 nm and assuming that 1 $O.D._{260}$ = 33 µg/ml of oligonucleotide solution.

2.5.13 Oligonucleotide Terminal Labelling

Synthetic oligonucletides were synthesized without a phosphate group at their 5' termini and labeled by transfer of the γ-^{32}P from [γ-^{32}P]ATP (Amersham, Buckinghamshire, UK) using the T4 polynucleotide kinase. 25 pm of oligonucleotide were labelled with 25 pm of [γ-^{32}P]ATP (3,000 Ci/mmol) in 15 µl of reaction mixture and 10U of T4 polynucleotide kinase (Boehringer Mannheim). The reaction was incubated at 37°C for 30 minutes and

blocked at 68°C for 10 minutes. The radiolabeled probe was purified from free [γ-^{32}P]ATP by gel-filtration chromatography through Sephadex G-25 (Pharmacia) with a Quick Spin™ column.

2.5.14 Random primer method for labelling DNA

Radioactive labelling of DNA fragments for hybridizations was performed according to the specifications for the random-primer kit of Boehringer Mannheim. Typically, 25 ng of isolated DNA to be labelled was dissolved in less than 10 µl of water and heat denatured at 95°C for 10 minutes. Immediately afterwards, it was quickly cooled on ice. The labelling reaction was prepared by adding 2 µl of reaction mix (containing a hexanucleotide mixture and 10X reaction buffer), 3 µl of dATP, dGTP, dTTP (0.5 µM), 50 µCi of [α–^{32}P] dCTP and 2U of Klenow and water to 20 µl. The reaction was then incubated at 37°C for 30 minutes. Afterwards, the reaction was terminated by heating the sample at 65°C for 10 minutes and passed over a Sephadex G-50 column to purify the probe from the unincorporated label.

2.5.15 Southern blot transfer

After electrophoresis the agarose gel was placed in 0.25 M HCl for 20 minutes, then rinsed in distilled water and immersed in denaturing solution (1.5 M NaCl, 0.5M NaOH) for 30 minutes. After denaturation the gel was rinsed, placed 15 minutes in neutralization solution (1.5 M NaCl, 0.5 M Tris-HCl pH 7.2, 0.001 M EDTA) and rinsed again. The gel was placed on a platform over three sheets of Whatman 3MM filter paper with their ends into a reservoir containing 20X SSPE (3.6 M NaCl, 0.2 M sodium phosphate, 0.02 M EDTA pH 8) and a nylon membrane Hybond™-N+ (Amersham), of the size of the gel, was placed over the gel excluding all the air bubbles by rolling with a pipette. Three more sheets of 3MM paper of the size of the gel were placed over the membrane and a final stack of absorbent paper towels was placed on the top. A 1 Kg weight was placed over the construction and transfer was allowed to proceed by capillarity over night. Afterwards, the filter was rinsed with 6X SSC and baked two hours at 30°C.

2.5.16 DNA hybridization

The filter was incubated for 1-2 hours with 0.2 ml/cm^2 of hybridization solution (5X SSPE, 5X Denhardt's solution, 10% w/v SDS). In the case a labeled oligonucleotide was used as a probe, the incubation was performed at 42°C; with longer DNA probes the temperature was ranging between 55-65°C depending on the level of homology. After pre-hybridization 1 ng/ml of hot probe was added and incubated overnight at room temperature. Filters hybridized with an oligo were washed extensively at room temperature with 6X SSC (1 M NaCl, 0.1 M Na_3citrate) and then 5-10 minutes or longer at the temperature of hybridization.

2.5.17 Dideoxy DNA sequencing method

The method of sequencing DNA was performed by the Sanger dideoxy method (Sanger *et al.*, 1977) utilizing the Sequenase (Version 2.0) DNA Sequencing Kit (USB, Cleveland, Ohio). Labelling reactions were performed using [α-^{35}S] ATP (>1000 Ci/mmol, Amersham). Universal M13/pUC forward and reverse primers were used for sequencing regions near vector sequences, while internal regions were sequenced by walking primers. All primers were synthesized by the ICGEB oligo synthetizer service

2.5.18 Polymerase chain reaction (PCR)

Polymerase chain reaction was performed using 2U Tth DNA polymerase (Boehringer Mannheim) in 100 µl of reaction volume. The reaction mixture contained 0.1-1 µg of DNA template, 0.2-1 µM of each primer, 2 µl of dATP, dGTP, dTTP, dCTP (10 mM) and 10 µl of 10X PCR buffer supplied by the manufacturer. It was overlayed with 100 µl of mineral oil. The reaction was incubated in a Thermocycler (Prem^{TM}, Lep Scientific, Milton Keynes, Great Britain) programmed for 30 cycles. Each cycle consisted of 1 minute denaturation at 95°C, 2 minute annealing at 55°C and 3 minutes of elongation at 72°C. A prolonged elongation time up to 7 minutes was used in the last cycle. DNA was then purified with the QIAquick Spin PCR Purification Kit (Qiagen).

2.6 Protein manipulation

2.6.1 Preparation of cell extracts

Cultures of *E. coli* or *B. pumilus* in LB plus *trans*-ferulic acid were monitored for the production of the enzyme by measuring the disappearance of the substrate at its maximal absorbance wavelength (310 nm). The growth was arrested when the degradation of the substrate was about 50% and the cells were collected by centrifugation at 8,000 rpm in a Sorvall GSA rotor. The cell pellet was washed twice with 50 mM Tris-HCl pH 7.2 and it was resuspended in 50% (w/v) 50 mM Tris-HCl pH 7.6, 10% (w/v) sucrose, 1 mM dithiotreitol, 1 mM EDTA, 0.1% Triton X100, 600 µg/ml lysozyme (Penalva and Salas, 1982). After incubation on ice for 1 hour the sample was frozen in dry ice, then thawed at 37°C for half an hour or till the suspension became clear.

2.6.2 Protein determination

Total protein concentration was determined using Bio-Rad Protein Assay, with bovine serum albumin as standard (Bradford, 1976)

2.6.3 Enzyme activity assay *in vitro*

The assay mixture was constituted by 50 mM sodium phosphate buffer (pH 6.0) and 0.5 g/l of ferulic acid. For testing the activity of the enzyme in anaerobiosis the mixture was degassed under vacuum and stored on ice for 4 hours in an anaeobic chamber (Coy Laboratory Products Inc., Grass Lake, Michigan) containing a 10% CO_2-10% H_2-80% N_2 atmosphere. Reactions were started by adding 5% v/v cell extract and incubating the mixture at 37°C for 1 hour in aerobiosis or anaerobiosis. In the second case, the assay mixture containing 0.025% Cysteine and 0.1 µg/ml of sodium resaruzin, was stabilized in anaerobic chamber until the color of the indicator faded after which the reaction was started. In both aerobiosis and anaerobiosis a control was introduced in which the assay mixture did not contain ferulic acid. Ferulate decarboxylase activity was assayed in aerobiosis and

anaerobiosis by monitoring the appearance of a new peak at 258 nm spectrophotometrically or by HPLC analysis.

2.6.4 Purification of recombinant *trans*-ferulic acid decarboxylase

The protein was purified from 8 liters of *E. coli* culture as described previously. All operations were carried out at 4°C. The chromatography procedures for the purification of FDC were performed on a low pressure liquid chromatography system (GradiFrac System, Pharmacia Biotech Norden AB, Sollentuna, Sweden). The cell extract was centrifuged at 28,000Xg for 1 hour to remove cell debris. Solid $(NH_4)_2SO_4$ was then added to the supernatant up to 20%, 40%, 60%, 80% saturation and centrifuged at 19,000 rpm for 30 minutes in a Sorvall SS-34 rotor. The pellet was dissolved in 2 ml of 20 mM Bis-Tris buffer pH 6, and enzyme activity was checked. Fractions having maximum activity were pooled. A Q Sepharose FF ion-exchange chromatography column (160x10 mm) (Pharmacia), equilibrated with 20 mM Bis-Tris pH 6.0, was loaded with the pooled fractions. After washing with two dead volumes of the same buffer, the proteins were eluted by a two-step gradient at a flow rate of 3 ml/min: 160 ml linear gradient from 0 to 400 mM NaCl in 20 mM Bis-Tris pH 6.0, followed by a 40 ml linear gradient from 400 mM to 1 M NaCl. Afterwards, the active fractions were pooled, the volume adjusted to 20 ml and the concentration of ammonium sulfate adjusted to 1.7 M. This sample was loaded onto a phenyl Sepharose HP hydrophobic interaction chromatography (HIC) column (160x10 mm) (Pharmacia), which had been equilibrated with 1.7 M $(NH_4)_2SO_4$ in 100 mM sodium phosphate buffer pH 7. Unbound proteins were washed off the column by 40 ml of the same buffer and bound proteins were eluted by 200 ml linear gradient from 1.7 M $(NH_4)_2SO_4$ in 100 mM sodium phosphate buffer pH 7 to 100 mM sodium phosphate buffer pH 7 at a flow rate of 3 ml/min. Active fractions were dialyzed against 20 mM Bis-Tris pH 6.0 and concentrated to 1 ml by Centricon 3 (Amicon, Inc. Beverly, Massachussetts) and finally purified through a gel filtration column (1x100 cm) of Sephacryl HR200 (Pharmacia) equilibrated with 20 mM Bis-Tris pH 6.0 at a flow rate of 0.5 ml/min. Fractions of 2.5 ml were collected and the enzymatic activity was recovered as a single peak.

2.6.5 Determination of native molecular mass of the enzyme

For the estimation of molecular mass a gel filtration column (1x100 cm) of Sephacryl HR200 (Pharmacia) was used. The system, equilibrated with 20 mM Bis-Tris pH 6.0, was run at a flow rate of 0.5 ml/min. Blue Dextran (2,000 KDa) was used to determine the void volume of the column and two protein markers, alcohol dehydrogenase (150 KDa) and carbonic anhydrase (29 KDa) (MWGF 200 Kit for molecular weight determination, Sigma, St. Louis, Missouri) were run through the column under the same conditions for calibration of the apparent molecular mass of the enzyme.

2.6.6 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Gels were prepared according to Laemmli (1970), except the concentration of acrylamide monomer stock solution was 29%:1%. As standard protein a low molecular weight calibration kit (Bio-Rad) with proteins having molecular weights from 94,000 to 14,400 was used. Electrophoresis was carried out for 3-4 h at constant 40 mA.

2.6.7 Isoelectric focusing

Analytical isoeletric focusing of the purified enzyme was carried out with an Ampholine PAGplate precast polyacrylamide gel (Pharmacia), with pH values ranging from 3 to 10, and the broad pI calibration kit as pI marker (Pharmacia).

2.6.8 High Performance Liquid Chromatography (HPLC)

Reversed-phase HPLC was performed on a Star 9010 solvent delivery system equipped with a Star 9050 detector connected to a computing integrator (model 4400; Varian Associates Inc., Walnut Creek, California) with Supelcosil LC8 or LC18 columns (Supelco Inc., Bellefonte, Pennsylvania).The system was run at a flow rate of 0.5 ml/min and methanol-H_2O was used as mobile phase. Culture supernatant samples were diluted in methanol, filtered and injected.

3. RESULTS

Section 1

3.1 Identification of rumen bacterial enzymes active on monomeric lignin aromatic compounds.

3.1.1 Introduction

Phenolic carboxylic acids occur widely in vascular plants, particularly widespread are substituted cinnamic acids (p-coumaric, ferulic and sinapic acids) closely related to the corresponding lignin monomers (p-coumaryl, coniferyl and sinapyl alcohol). In graminaceous plants, which represent a major component of ruminant diets, phenolic acids are common cell wall constituents esterified to the hemicellulose and partly responsible of the polysaccharide-lignin cross linkages. The relatively facile cleavage of these ester bonds, compared to the C-C and ether linkages in lignin, and the production of aryl esterases by rumen bacteria, suggests these phenolic compounds would be readily liberated in the rumen. In vitro studies have shown that these phenolic compounds are inhibitory toward rumen bacteria and are anti-quality factors in the forages. We investigated the ability of rumen microorganisms to modify these compounds.

3.1.2 Modification of phenolic compounds by strictly or facultatively anaerobic rumen bacteria

Eighteen different bacterial strains, not identified but just partially biochemically characterized, isolated in anaerobic atmosphere from rumen fluid of a fistulated cow fed with barley straw were tested for their ability to metabolize fourteen phenolic compounds: ferulic

Table 2. Biotransformation pattern of phenolic compounds by rumen bacteria.

STRAIN	SYRINGIC ACID	SYRINGIC ALDEHYDE	VERATRYL ALDEHYDE	*p*-HYDROXYBENZOIC ACID	VERATRIC ACID	SINAPIC ACID	BENZOIC ACID	PROTOCATECHUIC ACID	VINYLGUAIACOL	FERULIC ACID	*p*-COUMARIC ACID	*trans*-CINNAMIC ACID	VANILLIN	VANILLIC ACID
1	-	-	-	+	-	+	-	-	+	+	+	-	-	+
2	-	-	-	+	-	+	-	-	+	+	+	-	-	+
3	-	-	-	-	-	+	-	-	+	-	-	-	-	-
4	-	-	-	-	-	+	-	-	+	-	-	-	-	-
5	-	-	-	-	-	+	-	-	+	-	-	-	-	-
6	-	-	-	-	-	-	-	-	+	+	+	-	-	-
7	-	-	-	-	-	-	-	-	+	+	+	-	-	-
8	-	-	-	-	-	-	-	-	+	+	+	-	-	-
9	-	-	-	-	-	-	-	-	+	+	+	-	-	-
10	-	-	-	-	-	-	-	-	+	-	-	-	-	-
11	-	-	-	-	-	+	-	-	+	-	-	-	-	-
12	-	-	-	-	-	+	-	-	+	-	-	-	-	-
13	-	-	-	-	-	+	-	-	+	-	-	-	-	-
14	-	-	-	-	-	+	-	-	+	-	-	-	-	-
15	-	-	-	-	-	+	-	-	+	-	-	-	-	-
16	-	-	-	-	-	+	-	-	+	+	+	-	-	-
17	-	-	-	+	-	+	-	-	+	+	+	-	-	+
18	-	-	-	-	-	-	-	-	+	+	+	-	-	-

(+) modified UV spectrum; (-) unmodified UV spectrum

acid, *p*-coumaric acid, *trans*-cinnamic acid, sinapic acid, syringic acid, syringic aldehyde, veratryl aldehyde, veratric acid, benzoic acid, *p*-hydroxybenzoic acid, protocatechuic acid, vanillin, vanillic acid and vinylguaiacol (Table 2).

Each strain was grown in anaerobiosis for 48 hours, in thioglycollate medium containing 0.5 g/l of the interesting compound. According to UV spectrophotometric data none of the 14 aromatic compounds were completely mineralized anaerobically in the time of investigation. *Trans*-cinnamic acid, syringic acid, syringic aldhehyde, veratryl aldehyde, veratric acid, benzoic acid, vanillin and protocatechuic acid did not show any significant modification of their UV spectra. Twelve of the eighteen tested strains showed ability to modify similarly the UV spectrum of sinapic acid showing the appearance of a new peak at 269 nm and the disappearance of the peak at 304 nm. This activity was common among strictly as well as facultative anaerobe microorganisms. Transformation of ferulic acid by nine strains was strictly associated with the transformation of *p*-coumaric acid. In all these cases the appearance of new peaks at 258 nm and 256 nm respectively was monitored. While the UV spectrum of vanillin remained unmodified after 48 hours of incubation with all the strains analysed, the vanillic acid peak at 252 nm shifted to 269 after two days growth of three facultative anaerobic microorganisms, the same which showed activity toward *p*-hydroxybenzoic acid (shift of the characteristic peak from 246 nm to 267 nm). All four strains were also able to degrade sinapic, ferulic and *p*-coumaric acid. The concentration of vinylguaiacol was slightly reduced in the medium after incubation and this phenomenon did not correlate with the appearance of new absorption peaks. This could be explained by the uptake of this aromatic compound by the cells or by the high volatility of this compound.

3.1.3 Biotransformation of hydroxycinnamic compounds by *Bacillus* spp.

B. pumilus PS213 was isolated in our laboratory from a microbial consortium of the cow rumen fluid. This consortium was grown aerobically in minimal media containing 50 mg % (w/v) of the selective substrates *trans*-cinnamic acid, *p*-coumaric acid and ferulic acid. Since

the growth was very slow 2 g/l casamino acid were added. After several transfers in fresh medium the consortium lost its ability to degrade *trans*-cinnamic acid, however *p*-coumaric acid and ferulic acid degradation was successfully maintained. Bacteria able to convert these substrates to different products were isolated and the most active one was found to be a strictly aerobic microorganism, successively identified as *Bacillus pumilus*. This microorganism was also able to modify sinapic acid. *Bacillus pumilus* could not grow on minimal media (without casamino acid) containing sinapic, ferulic and *p*-coumaric acid, therefore it could not utilize any of these compounds as sole carbon source.

We tested other *Bacillus* species commonly isolated from the rumen for their ability to degrade hydroxycinnamic acids. *B. subtilis* 1012, which is not a common guest of the rumen, was also introduced in the test. This analysis *in vivo* was performed as previously described under aerobic conditions in LB medium containing ferulic, *p*-coumaric, sinapic and *trans*-cinnamic acid respectively. Modifications of the UV spectra of these substrates were monitored spectrophotometrically at several intervals of growth up to 5 days. As shown in Table 3 all *Bacillus* strains tested were able to modify these compounds, except for *trans*-cinnamic acid, generating the same product from each substrate.

Table 3. Biotransformation of cinnamic acids by *Bacillus* spp.

STRAIN	FERULIC ACID	*p*-COUMARIC ACID	SINAPIC ACID	*t*-CINNAMIC ACID
B. pumilus PS213	+	+	+	—
B. licheniformis LMG 6933	+	+	+	—
B. circulans LMG 6926	+	+	+	—
B. laterosporus LMG 6931	+	+	+	—
B. macerans LMG 6324	+	+	+	—
B. subtilis 1012	+	+	+	—

3.1.4 Decarboxylase activity in *Bacillus* spp.

Following the shift in UV absorption of the maximum wavelength of ferulic acid and *p*-coumaric acid, it was suggested that the resulting products could be 4-vinylguaiacol and 4-vinylphenol respectively. Initially, to confirm the transformation of ferulate to 4-vinylguaiacol by *Bacillus pumilus*, we compared by HPLC analysis the elution time of a 4-vinylguaiacol standard and that of the unknown peak of the supernatant of a *B. pumilus* overnight culture in M9CA medium containing ferulic acid (Fig. 5). The elution time were exactly the same confirming the identity of the spectra and the strong smell characteristic of the product. Moreover, we demonstrated by HPLC the identity between the peak of 4-vinylphenol and the unknown peak of a *B. pumilus* culture grown in presence of *p*-coumaric acid (Fig. 6). These conversions provided evidence of a ferulate and *p*-coumarate decarboxylase activity in *B. pumilus*. (Fig. 7). Though a modification of the spectrum for sinapic acid was observed, it was not possible to demonstrate by HPLC its decarboxylation to 3,5-dimethoxy-4-hydroxy styrene because this standard is not commercially available.

The wide distribution of this decarboxylase activity among *Bacillus* strains, was confirmed by the observation of the same modification of the UV spectra of ferulic and *p*-coumaric acid respectively, after an overnight growth of *B. pumilus* PS213, *B. licheniformis* LMG 6933, *B. circulans* LMG 6926, *B. laterosporus* LMG 6931, *B. macerans* LMG 6324 and *B. subtilis* 1012 in rich medium (LB) containing each substrate.

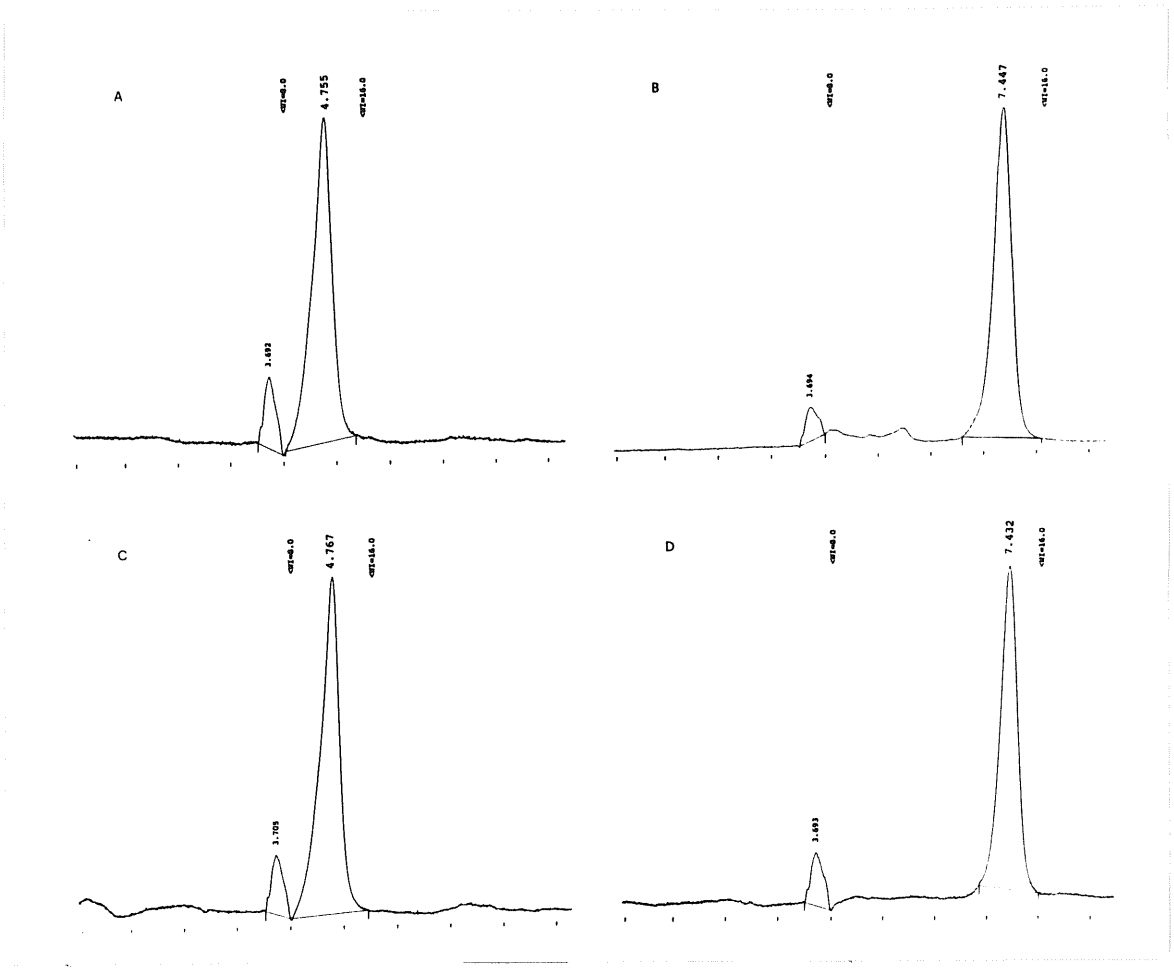


Fig. 5. HPLC retention time of ferulic acid (**A, C**) and 4-vinylguaiacol (**B, D**) at λ=260 nm and flow rate = 0.5 ml min^{-1}. (**A**) Ferulic acid 0.5 µg ml^{-1} standard; (**B**) 4-vinylguaiacol 0.5 µg ml^{-1} standard; (**C**) M9CA medium plus 0.5 µg ml^{-1} ferulic acid; (**D**) M9CA medium plus 0.5 µg ml^{-1} ferulic acid after an overnight growth of *B. pumilus* (Varian LC Star Program).

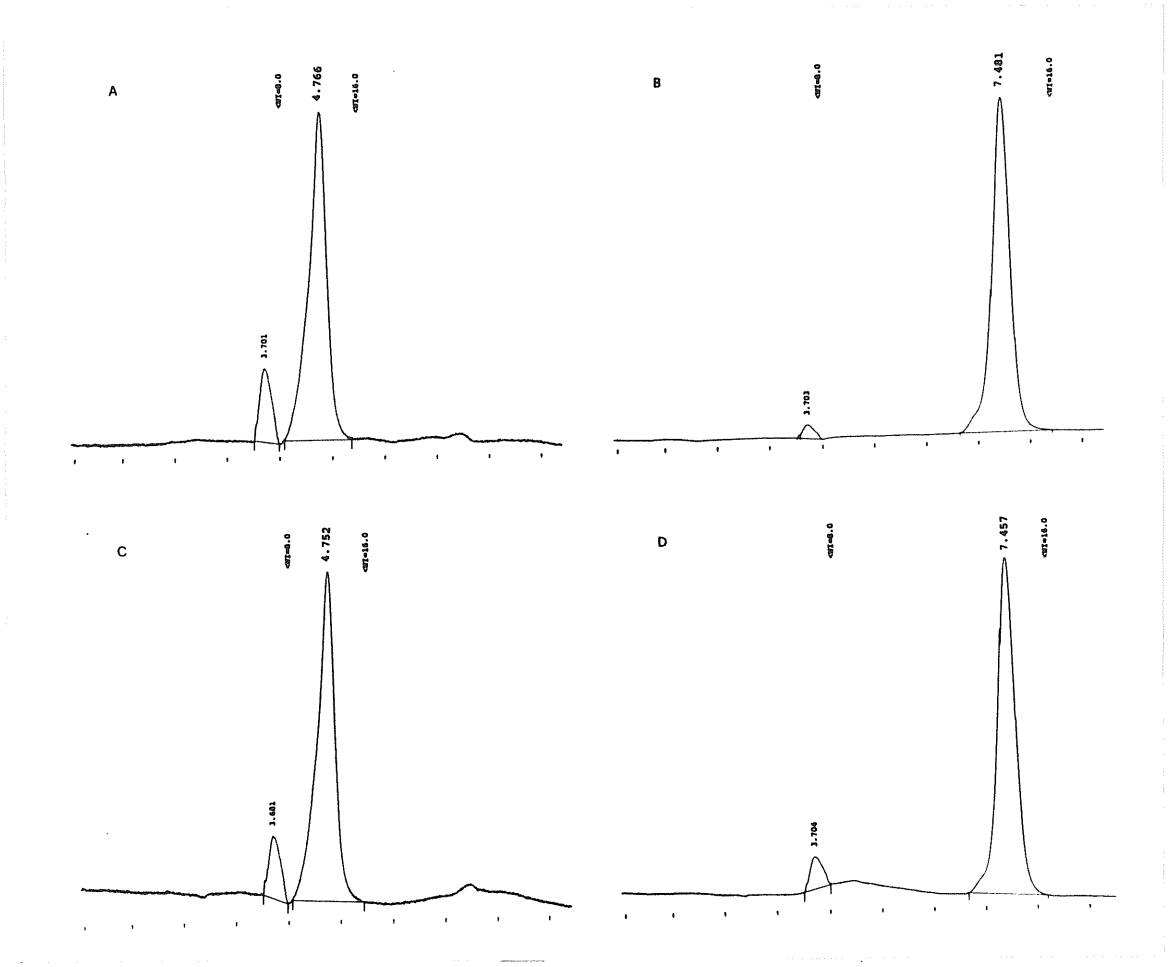
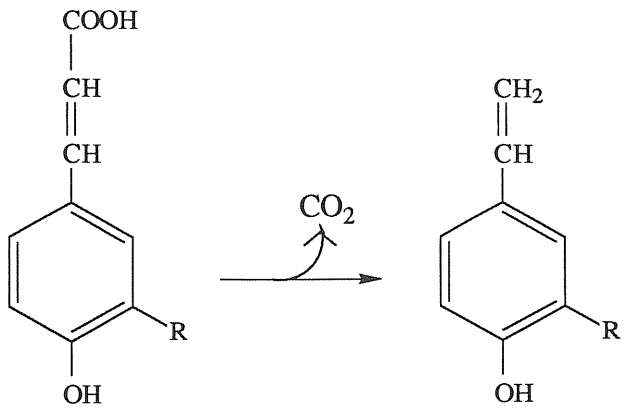


Fig. 6. HPLC retention time of *p*-coumaric acid (**A, C**) and 4-vinylphenol (**B, D**) at λ=260 nm and flow rate = 0.5 ml min^{-1}. (**A**) *p*-coumaric acid 0.5 µg ml^{-1} standard; (**B**) 4-vinylphenol 0.5 µg ml^{-1} standard; (**C**) M9CA medium plus 0.5 µg ml^{-1} *p*-coumaric acid; (**D**) M9CA medium plus 0.5 µg ml^{-1} *p*-coumaric acid after an overnight growth of *B. pumilus* (Varian LC Star Program).



R = OCH_3 (Ferulic acid) R = OCH_3 (4-Vinylguaiacol)

R = H (*p*-Coumaric acid) R = H (4-Vinylphenol)

Fig. 7. Ferulate and *p*-coumarate decarboxylase activity

The ferulate decarboxylase activity was detected in crude cell-free extract prepared from *B. pumilus* PS213 grown overnight in LB medium plus 0.5 g of ferulic acid per liter. The modification of the UV spectrum of the compound in the assay mixture, after incubation in aerobiosis at 37°C, was followed observing that it changed as in the whole cell-containing culture. The result was confirmed by HPLC analysis showing the appearance of a new peak having the retention time of 4-vinylguaiacol (Fig. 8). On the contrary no activity was detected in the supernatant of the *B. pumilus* PS213 culture. Similar results were obtained in our laboratory performing the same test with crude cell-free extract of *B. licheniformis* LMG 6933.

3.1.5 Non-oxidative decarboxylation

To check if the enzyme was able to work also in anaerobiosis the ferulate decarboxylase activity was tested in crude cell-free extract of a *B. pumilus* PS213 culture, grown in LB containing 0.5 g/l ferulic acid, degassed to eliminate O_2 and added to the reaction mixture stabilised in an anaerobic chamber. The conditions of anaerobiosis were ensured also after having added the cell extract. The decarboxylase was able to work in the absence of O_2, as shown by spectrophotometer and HPLC analysis (Fig. 9).

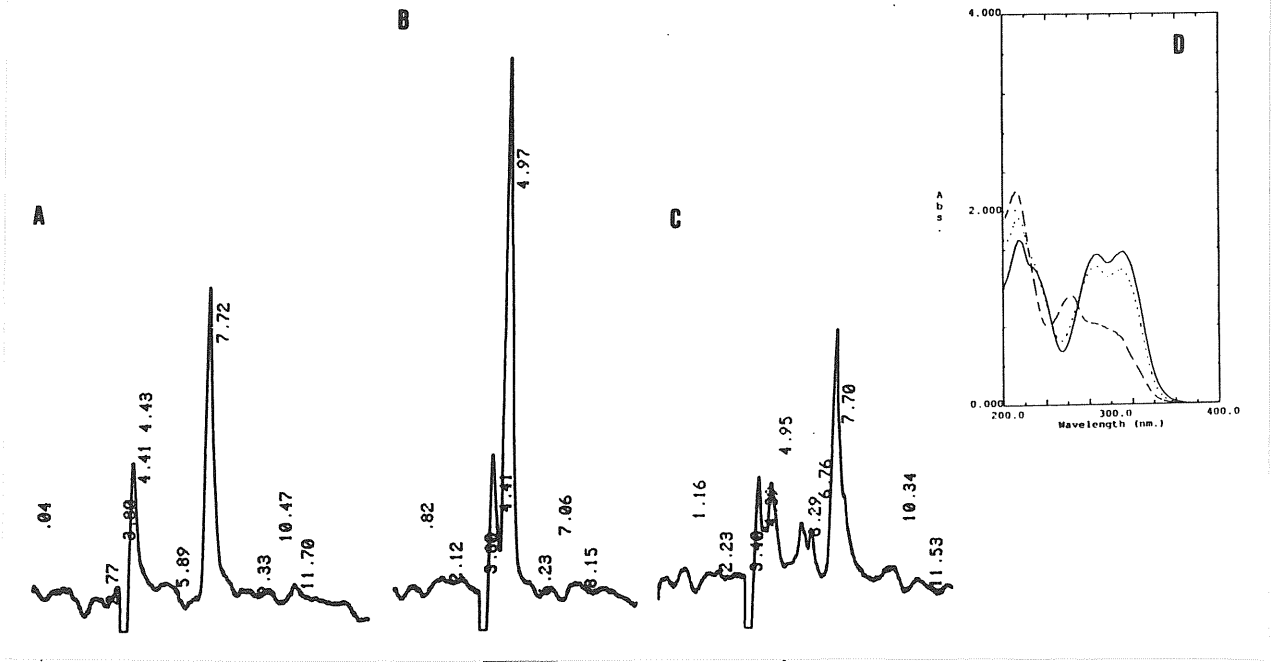


Fig. 8. HPLC retention time of 0.5 µg ml^{-1} 4-vinylguaiacol (**A, C**) and 0.5 µg ml^{-1} ferulic acid (**B**) at λ=260 nm and flow rate = 0.5 ml min^{-1}. (**A**) 4-vinylguaiacol 0.5 µg ml^{-1} standard; (**B**) ferulic acid 0.5 µg ml^{-1} standard; (**C**) 50 mM phosphate buffer pH 6 plus ferulic acid after incubation with *B. pumilus* cell extract in aerobiosis (Varian integrator model 4400). (**D**) UV spectra of ferulic acid after incubation in aerobiosis with cell extract of *B. pumilus* grown in LB plus ferulic acid (- - -) or in LB without ferulic acid (...); control (—).

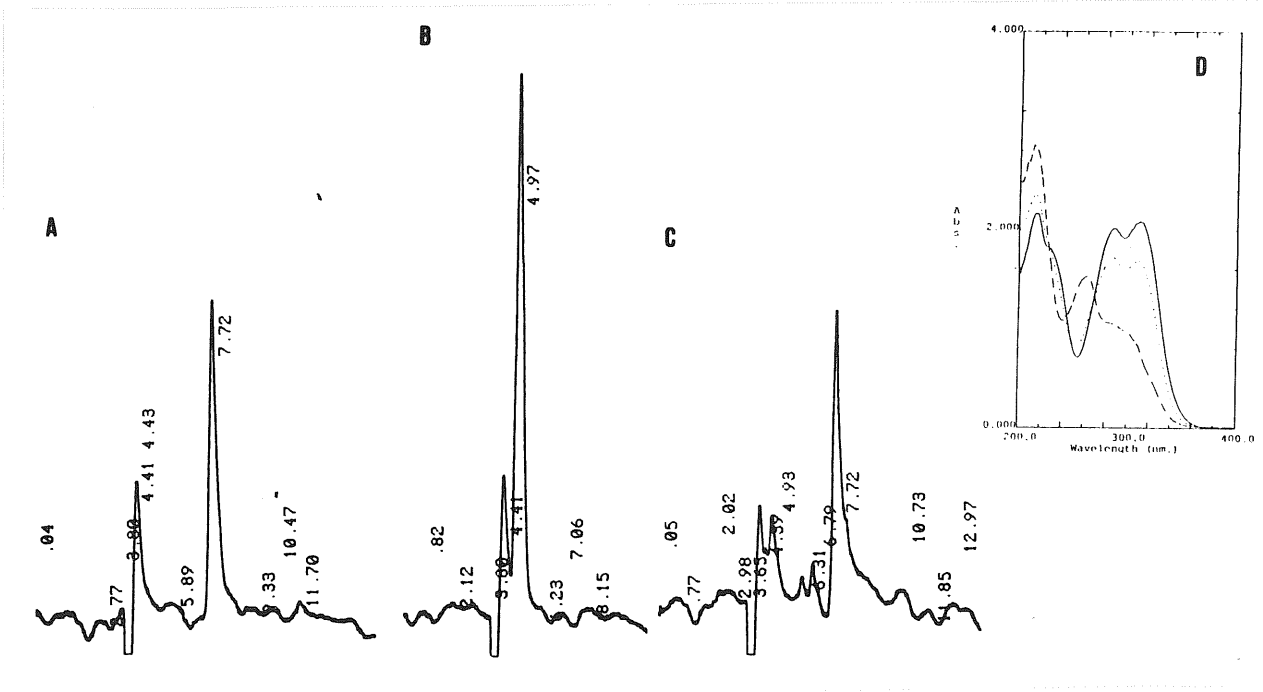


Fig. 9. HPLC retention time of 4-vinylguaiacol (**A, C**) and ferulic acid (**B**) at λ=260 nm and flow rate = 0.5 ml min^{-1}. (**A**) 4-vinylguaiacol 0.5 µg ml^{-1} standard; (**B**) ferulic acid 0.5 µg ml^{-1} standard; (**C**) 50 mM phosphate buffer pH 6 plus ferulic acid after incubation with *B. pumilus* cell extract in anaerobiosis (Varian integrator model 4400). (**D**) UV spectra of ferulic acid after incubation in anaerobiosis with cell extract of *B. pumilus* grown in LB plus ferulic acid (- - -) or in LB without ferulic acid (...); control (—).

3.1.5 Inducibility of the decarboxylase activity

Inducibility of the decarboxylase activity in *B. pumilus* was tested as described in material and methods. The cells grown overnight in the presence or absence of ferulic acid ($O.D._{600}$ 2.61 and 2.71 respectively) were centrifuged and resuspended in LB plus this compound to the same $O.D._{600}$ of 2.3. After only 10 minutes a significant difference in the rate of decarboxylation of ferulic acid by the induced culture in comparison to the non induced one was observed. There was little difference between the UV spectra of the supernatant of two cultures in the successive three measurements made every 20 minutes, while the optical density was increasing very slowly, confirming that the culture was still in the lag phase. After about two hours the optical density of the two cultures at 600 nm reached 3 and the two spectra overlapped (Fig. 10). The inducibility of the ferulate decarboxylase activity was detected also for *B. licheniformis* LMG 6933 and *B. subtilis* 1012 (data not shown).

The inducibility was further demonstrated incubating in phosphate buffer plus ferulic acid cell extracts of *B. pumilus PS213* grown in presence and absence of ferulic acid. Although the decarboxylase activity is inducible, a low constitutive activity was detected in the cell free extract from a culture of *B. pumilus* PS213 grown in the absence of ferulic acid and incubated with the enzyme assay reaction mixture in aerobiosis and anaerobiosis (Fig. 8D, Fig. 9D).

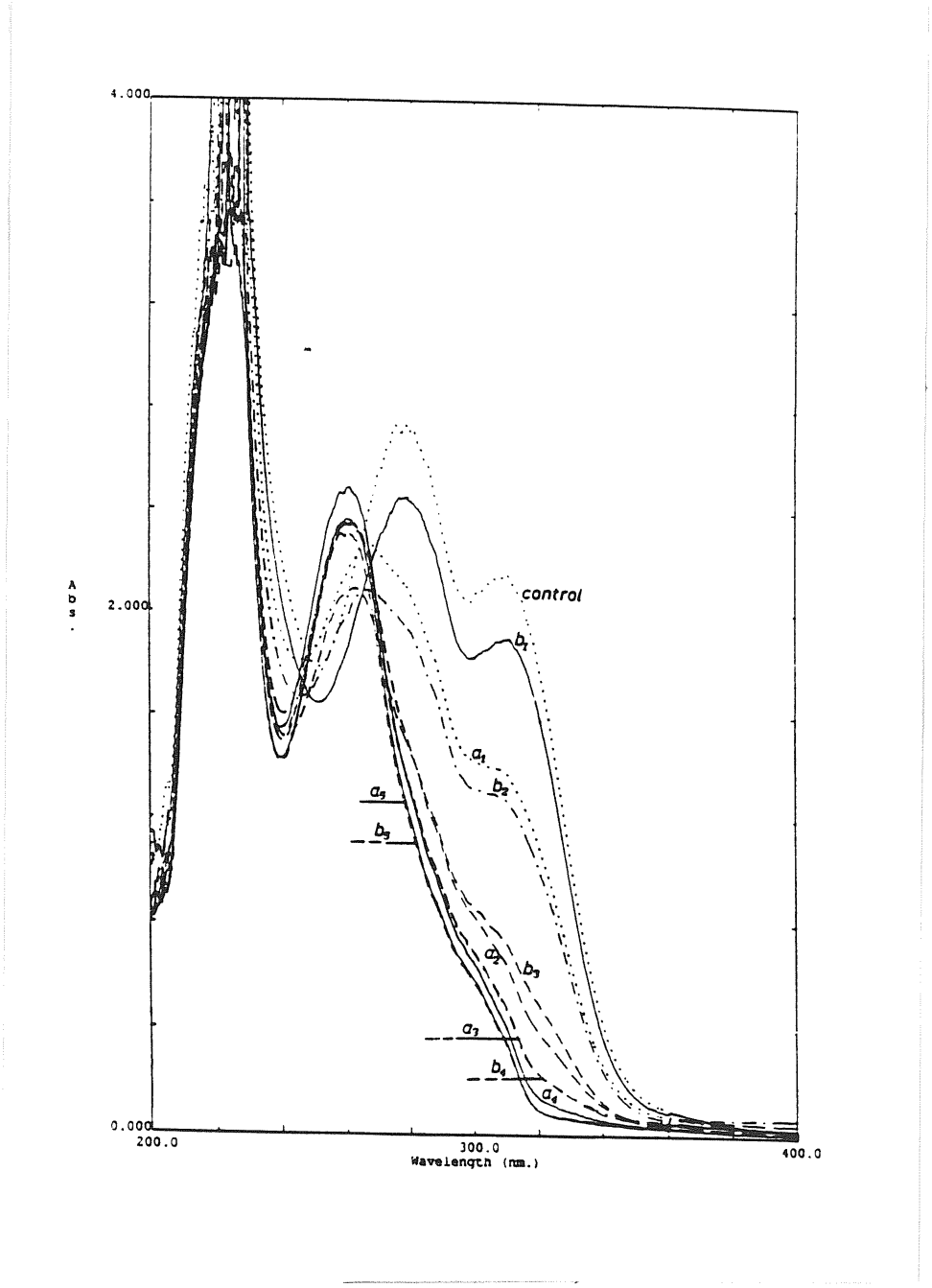


Fig. 10. Test of inducibility of the decarboxylase activity. Control is LB plus 0.5 mg ml^{-1} of ferulic acid; **(a)** decarboxylation of ferulic acid by the induced culture and **(b)** non induced culture after incubation at 37°C for 10' (1), 30' (2), 50' (3), 70' (4) and 130' (5).

Section 2

3.2 Cloning, sequencing, and expression in *Escherichia coli* of the *Bacillus pumilus* gene for ferulic acid decarboxylase (*fdc*)

3.2.1 Introduction

The decarboxylation of ferulic acid has already been described for several microorganisms (Healey et al., 1980; Andreoni et al., 1984; Arfman and Abraham, 1989; Clausen et al., 1993; Huang et al., 1993); and the enzyme has been purified from Pseudomonas fluorescens (Huang et al., 1994) and in our laboratory from Bacillus pumilus (Degrassi et al., 1995). However, the genes encoding these functions have not been investigated.

3.2.2 Cloning of ferulic acid decarboxylase gene from *B. pumilus*

The N-terminal amino acid sequence of the *trans*-ferulic acid decarboxylase, previously purified in our laboratory from *B. pumilus* (Degrassi *et al.*, 1995), was analyzed by automated pulse liquid-phase Edman degradation. The N-terminal sequence was identify as follows:

H_2N-Met-Asp-Gln-Phe-Val-Gly-Leu-His-Met-Ile-Tyr-Thr-Tyr-Gln-Asn-Gly-Val...

A 51-mer oligonucleotide was designed on the basis of this amino acid sequence using the program BackTranslate (WU-GCG) with the codon frequencies of *Bacillus subtilis* (105 genes found in GenBank 63). The ^{32}P-labelled 51 mer oligonucleotide 5' ATGGATCAATTTGTTGGCCTTCATATGATTTATACA TATGAAAATGGCGT 3' was used as a probe in a Southern blot analysis of *B. pumilus* genomic DNA digested to completion with *Eco*RI, *Pst*I, *Hin*dIII, *Bam*HI, *Bcl*I and *Sac*I restriction enzymes. Bands of approximately 3.5 Kb were shown by

*Eco*RI and *Bcl*I digestions; DNA cut with *Bam*HI, *Sac*I and *Pst*I gave bands of about 7 Kb, 10 Kb and 30 Kb respectively, while *Hin*dIII digestion generated a band of approximately 4 Kb. As a negative control, the probe did not hybridize with total DNA extracted from *E. coli* DH5α (Fig. 11).

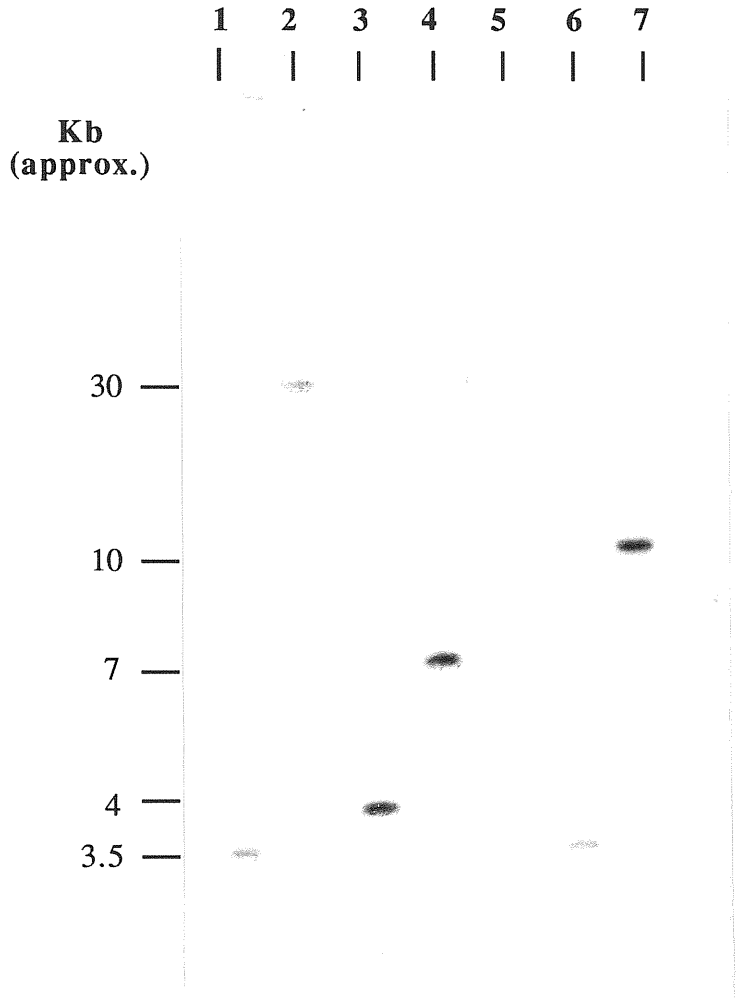


Fig. 11. Digestion of B. pumilus chromosomal DNA with the restriction enzymes **(1)** *Eco*RI, **(2)** *Pst*I, **(3)** *Hin*dIII, **(4)** *Bam*HI, **(6)** *Bcl*I, **(7)** *Sac*I. DH5α total DNA as negative control **(5)**.

*Hin*dIII DNA fragments ranging between 3.5 and 4.5 Kb were eluted from agarose gel and ligated into the *Hin*dIII restriction site of pUC19 to construct a sublibrary in *E. coli* DH5α. Screening of this sublibrary allowed the identification of a positive clone. *Hin*dIII digestion of plasmidic DNA extracted from this clone and its subsequent Southern blot analysis showed that it contained three different inserts, of which only

one was able to hybridize with the probe. This positive fragment of 3,690 bp was subcloned into pUC19 and transformed again into *E. coli* DH5α. Since this fragment conferred to the host strain the ability to decarboxylate *trans*-ferulic acid *in vivo* after an overnight growth in LB medium plus ferulic acid, we assumed that it contained the entire gene encoding the *trans*-ferulic acid decarboxylase. No decarboxylation was observed in an overnight culture of *E. coli* DH5α and *E. coli* DH5α containing pUC19 alone (Fig. 12).

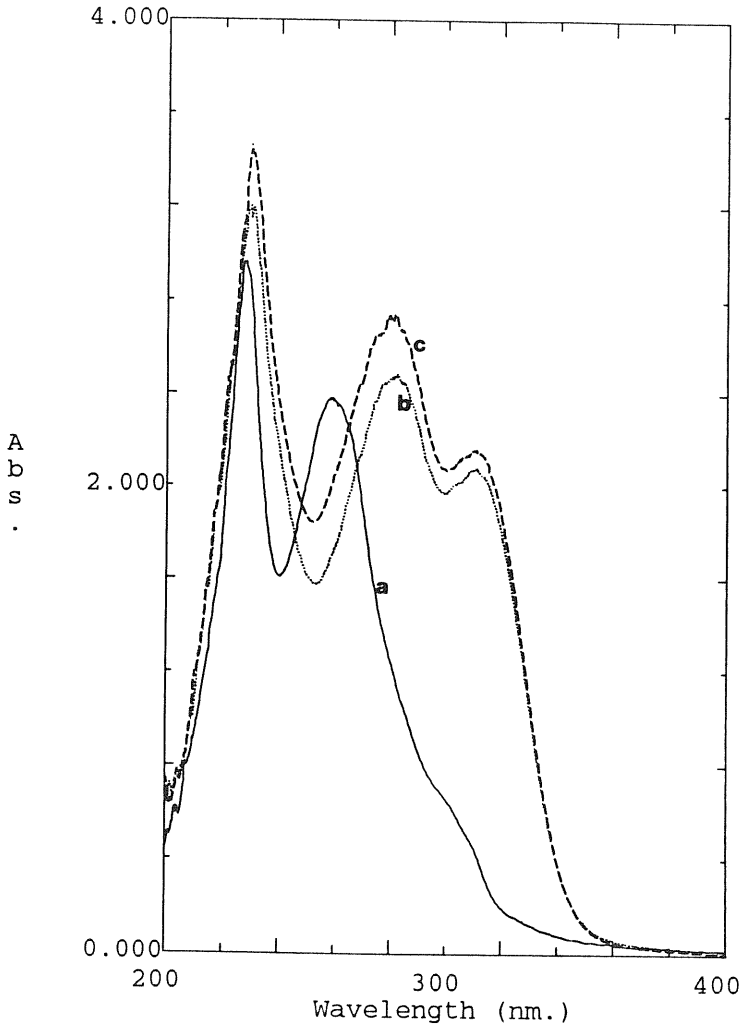


Fig. 12. Absorption spectra of : **(a)** an overnight culture of *E. coli* DH5α containing the *fdc* gene; **(b)** an overnight culture of the host strain *E. coli* DH5α. **(c)** LB medium plus 0.5 g/l of ferulic acid utilized as inoculum medium.

HPLC analysis showed that this fragment not only conferred to *E. coli* DH5α the ability to decarboxylate ferulic acid to 4-vinylguaiacol (Fig. 13), but also *p*-coumaric acid to 4-vinylphenol (Fig. 14). However, the DNA fragment did not seem to be responsible for the modification of sinapic acid in *B. pumilus*, since a comparison between the chromatograms of the supernatant of the clone with the insert and the DH5α strain without the insert did not show any significant difference.

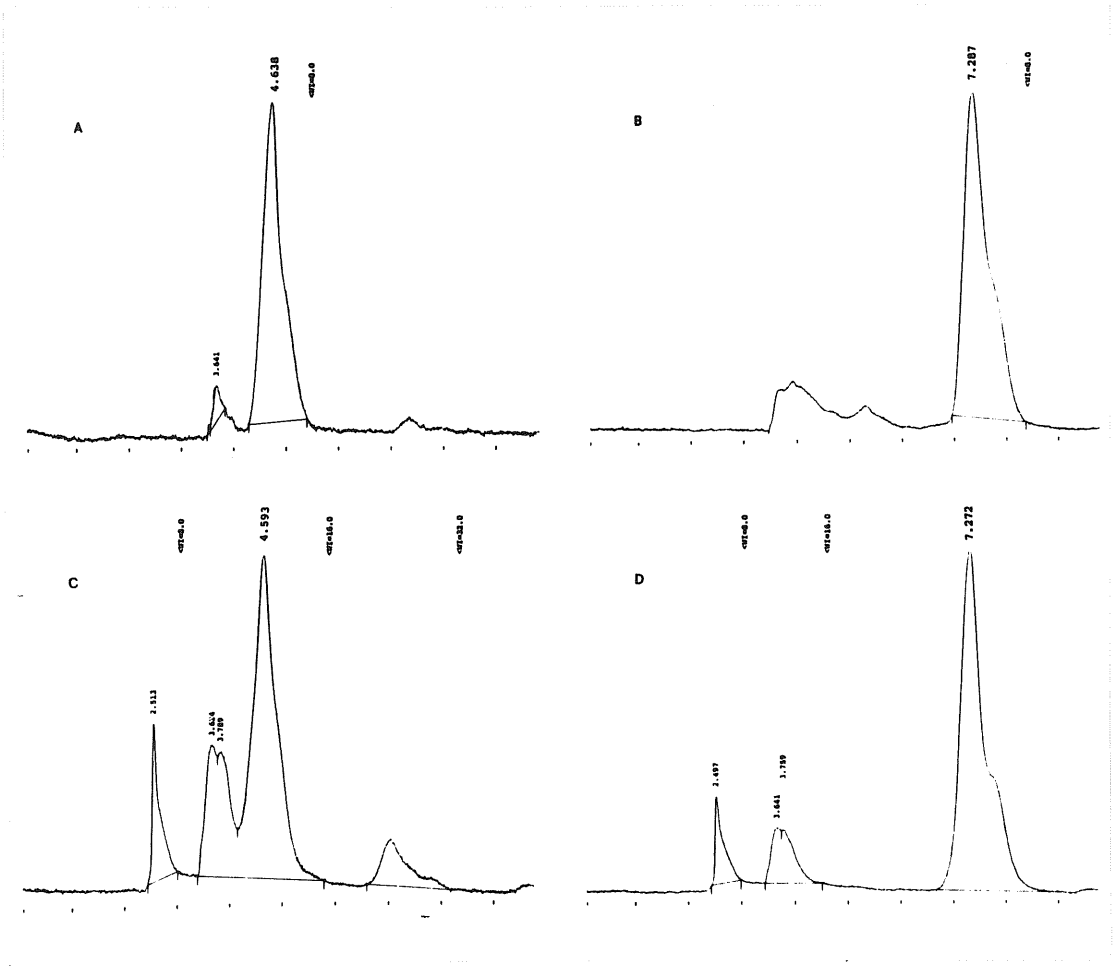


Fig. 13. HPLC retention time of ferulic acid (**A, C**) and 4-vinylguaiacol (**B, D**) at λ=260 nm and flow rate = 0.5 ml min^{-1}. (**A**) Ferulic acid 0.5 μg ml^{-1} standard; (**B**) 4-vinylguaiacol 0.5 μg ml^{-1} standard; (**C**) LB medium plus 0.5 μg ml^{-1} ferulic acid and 100 μg ml^{-1} ampicillin after an overnight growth of *E. coli* DH5α harboring pUC19; (**D**) LB medium plus 0.5 μg ml^{-1} ferulic acid and 100 μg ml^{-1} ampicillin after an overnight growth of *E. coli* DH5α harboring *fdc* gene (Varian LC Star Program).

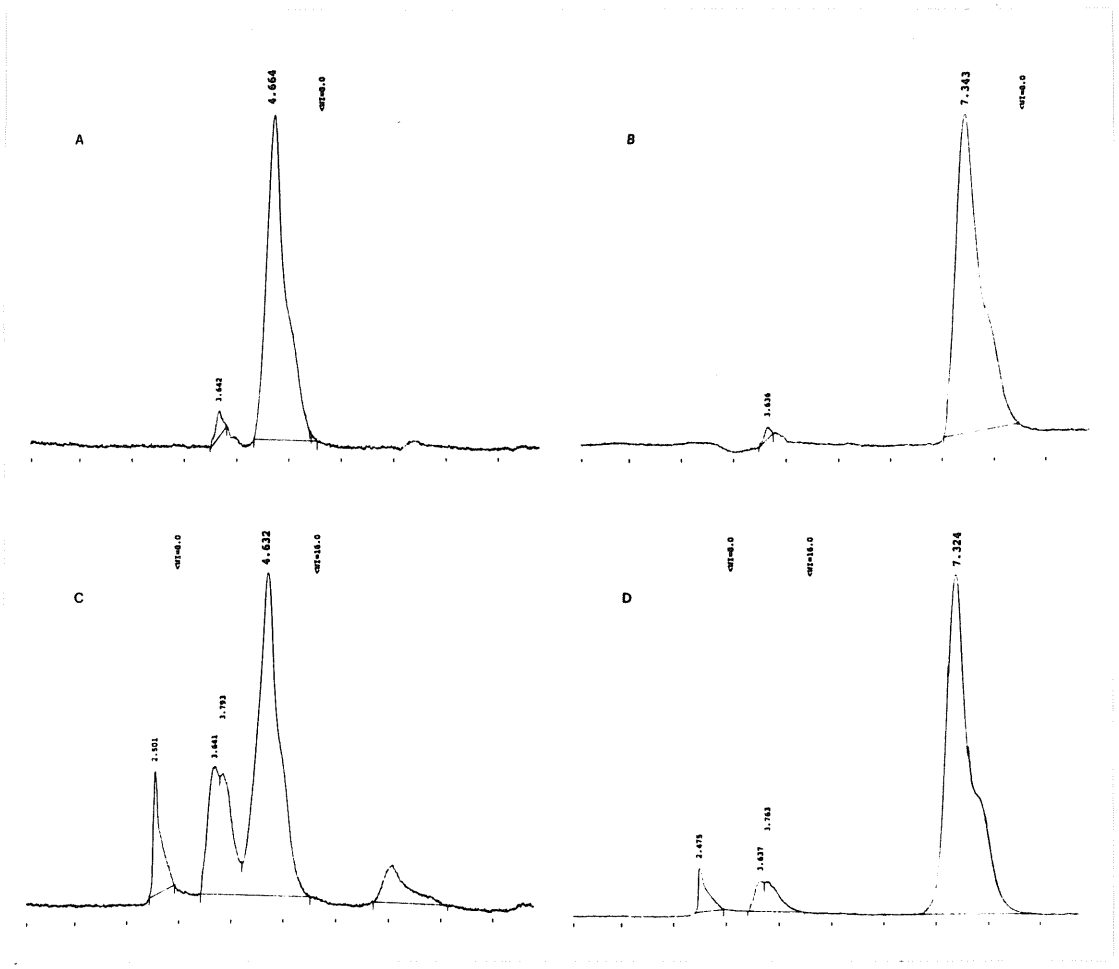


Fig. 14. HPLC retention time of *p*-coumaric acid (**A, C**) and 4-vinylphenol (**B, D**) at λ=260 nm and flow rate = 0.5 ml min^{-1}. (**A**) *p*-coumaric acid 0.5 μg ml^{-1} standard; (**B**) 4-vinylphenol 0.5 μg ml^{-1} standard; (**C**) LB medium plus 0.5 μg ml^{-1} *p*-coumaric acid and 100 μg ml^{-1} ampicillin after an overnight growth of *E. coli* DH5α harboring pUC19; (**D**) LB medium plus 0.5 μg ml^{-1} *p*-coumaric acid and 100 μg ml^{-1} ampicillin after an overnight growth of *E. coli* DH5α harboring *fdc* gene (Varian LC Star Program).

3.2.3 Deletion analysis and DNA sequence of *B. pumilus* ferulic acid decarboxylase (*fdc*) gene.

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. For this purpose a physical map of the insert was determined (Fig. 15). No *Nhe*I, *Xho*I, *Sal*I, *Sma*I, *Bgl*II, *Sac*I, *Not*I, *Bam*HI restriction sites were detected. According to the information of the map, deletions were made with *Cla*I, *Eco*RI and *Xba*I restriction enzymes and plasmids pUCFDCΔ1, pUCFDCΔ2, pUCFDCΔ3, pUCFDCΔ4 and pUCFDCΔ5 were inserted into *E. coli* DH5α and transformed clones were subjected to *in vivo* assay for ferulic acid decarboxylase activity. Fig. 15 summarizes the results of the deletion analysis. The 1,330 bp *Hin*dIII-*Xba*I fragment contained in the deletion plasmid pUCFDCΔ2 encoded all the necessary information for the expression of the decarboxylase activity. This fragment, and 350 bases downstream the unique *Xba*I site in the 3,690 bp insert of pUCFDC were sequenced on both strands and the sequence was analyzed. A 486 bp open reading frame (ORF) was found starting at an ATG codon at nucleotide 816 and extending to a TAG codon at nucleotide 1301. It encodes a deduced 19,069 Da polypeptide consisting of 161 amino acid residues (Fig. 16).

The nucleotide sequence at the 5' end of this ORF 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* (not shown). The only difference is a Trp-17 instead of a Val-17. A comparative search with the nucleotide sequence of *fdc* gene in GenBank and EMBL as well as with the deduced polypeptide in SwissProt, PIR and GenePept data banks by the BLAST Mail-Server program did not show any interesting homology. The yeast phenylacrylic acid decarboxylase (PAD1), which decarboxylate ferulic acid (Clausen *et al.*, 1994) (GenBank accession number P33751), shows only a 66.7% identity in a 6 amino

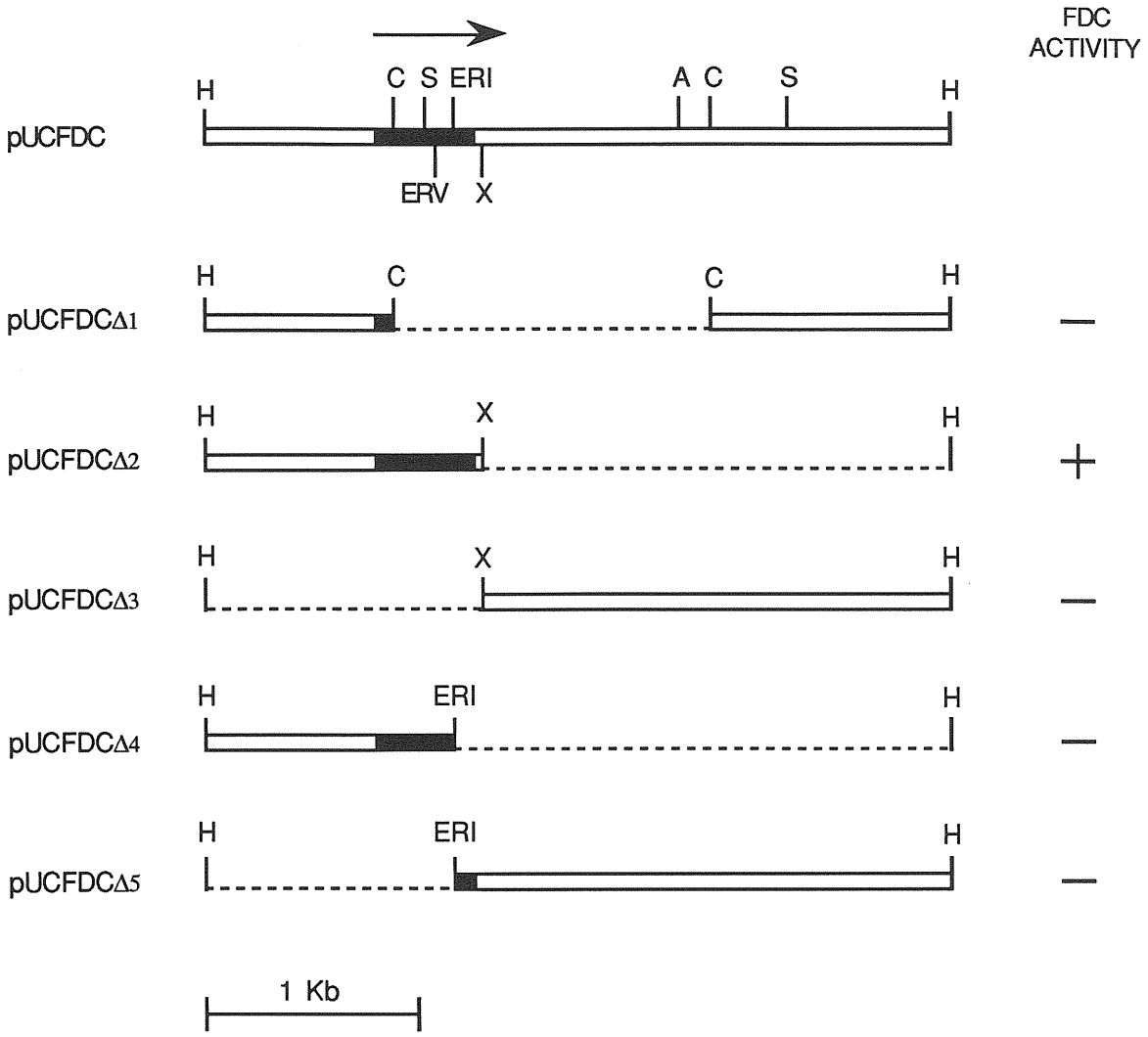


Fig. 15. Restriction enzyme map of the 3,690 bp *Hin*dIII fragment inserted into pUC19 (pUCFDC). A, *Ava*I; C, *Cla*I; ERI, *Eco*RI; ERV, *Eco*RV; H, *Hin*dIII;. S, *Sph*I; X, *Xba*I. The deletion plasmids pUCFDCΔ$_1$, pUCFDCΔ$_2$, pUCFDCΔ$_3$, pUCFDCΔ$_4$, pUCFDCΔ$_5$, made with *Cla*I, *Xba*I and *Eco*RI, are shown. On the right, the ferulate decarboxylase (FDC) activity of the various transformants with the deletion plasmids is reported.

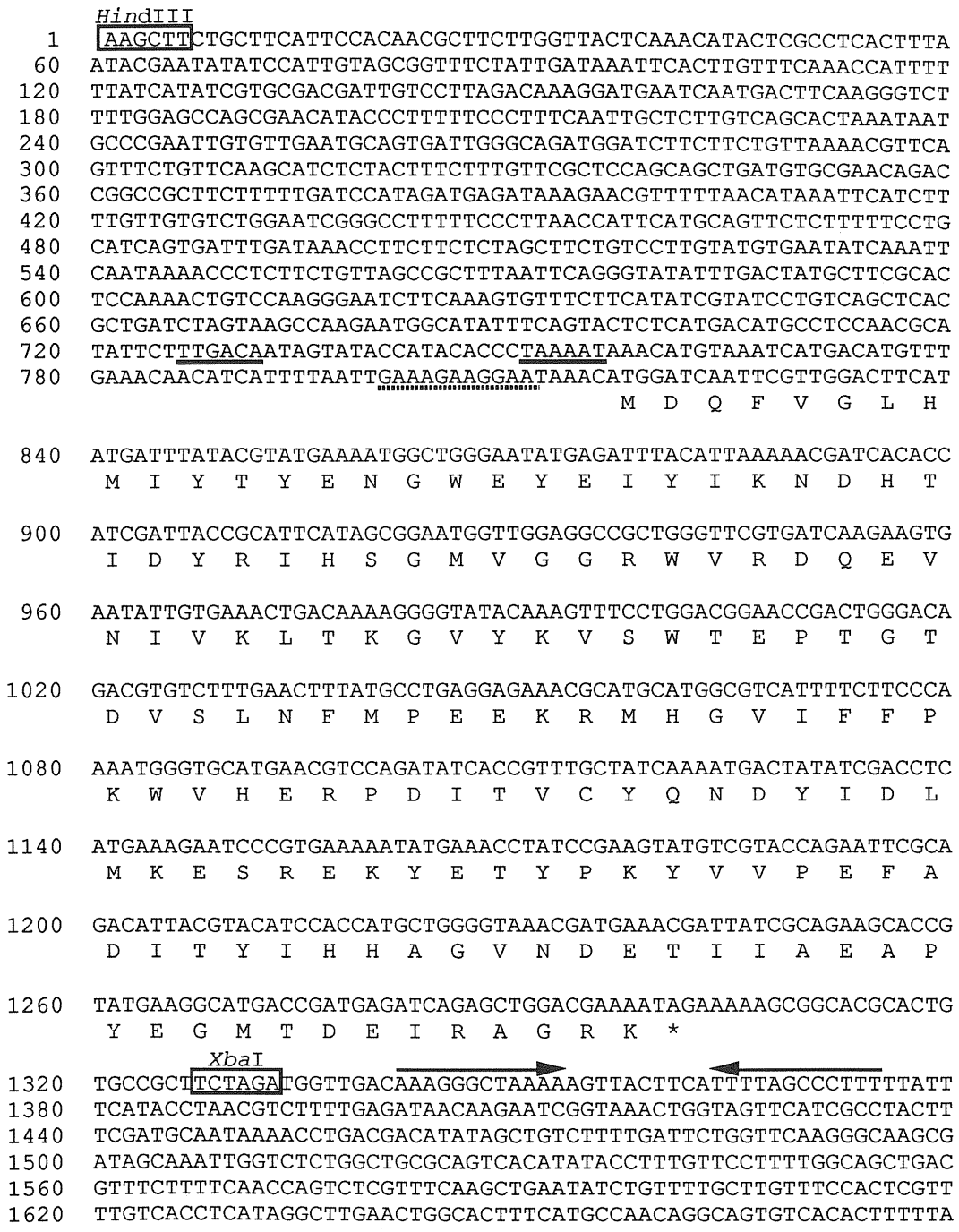


Fig. 16. Nucleotide sequence of the *fdc* gene. Nucleotides are numbered from the 5' end, and the predicted amino acid sequence for the *fdc* gene is given in single-letter code below the DNA sequence. A possible promoter sequence is underlined; a putative Shine-Dalgarno region is shown (dotted line). Opposing arrows indicate inverted repeats of a possible rho-independent terminator.

acids overlap with the *B. pumilus* ferulate decarboxylase.

A putative Shine-Dalgarno sequence was observed 5 bp upstream the initiation codon ATG. A potential promoter sequence TAAAAT, which is similar to the consensus -10 procaryotic promoter TATAAT, between nucleotides 751 and 756 and an *E. coli* -35 consensus sequence TTGACA were found. The spacing between the -10 and -35 elements is 18 bp, near to the optimal 17 bases distance (Ogasawara, 1985). Also 39 base pairs downstream the termination codon there is a sequence resembling a rho-independent terminator. A 645 bp ORF of unknown significance was observed on the complementary DNA strand in the opposite orientation, starting 27 nucleotides 5' from the ATG of *fdc*.

3.2.4 Purification and characterization of recombinant ferulic acid decarboxylase.

Table 4 summarizes the purification procedure of the ferulate decarboxylase expressed in *E. coli*. The purity of the decarboxylase preparation was demonstrated by SDS-PAGE (Fig. 17) and HPLC (data not shown). 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 mmol 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-PAGE 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* (Degrassi *et al.*, 1995). The isoelectric point determined by means of isoelectric focusing is 4.8 (Fig. 18), very near to the value measured for the enzyme purified from *B. pumilus* (Degrassi *et al.*, 1995).

Table 4. Purification of *B. pumilus* ferulate decarboxylase expressed in *E. coli* [a]

Purification step	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification Factor	Yield (%)
Cell extract	510	218.45	0.428	1	100
$(NH_4)_2SO_4$	308	161.57	0.524	1.22	74
Q Sepharose FF	23.75	89.3	3.76	8.78	40.8
Phenyl Sepharose	0.472	14.8	31	72.4	6.7
Sephacryl HR 200	0.106	9.4	88	205.6	4.3

[a] A 8-liter sample of a 12-h culture was purified. For details, see Materials and Methods

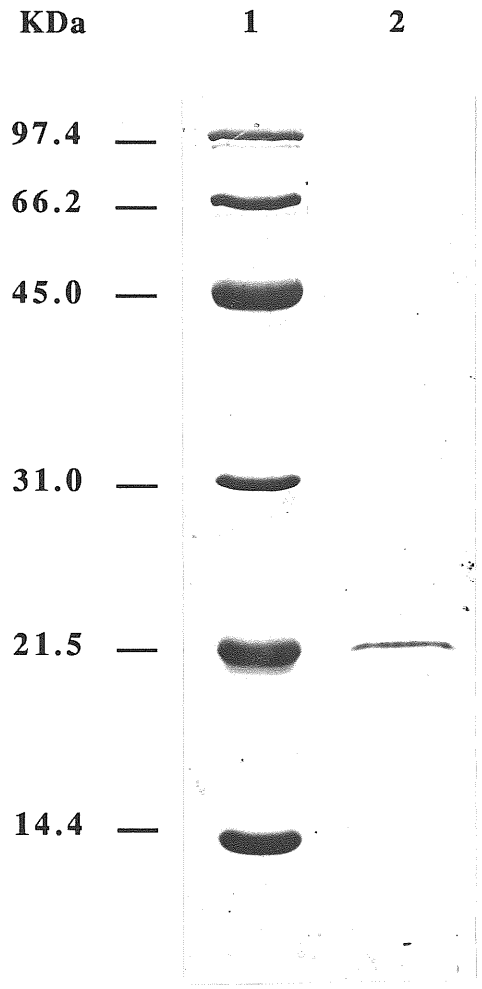


Fig. 17. SDS-PAGE of the ferulate decarboxylase purified from *E. coli*. Lanes: 1, molecular mass standards; 2, peak fraction from the Sephacryl HR200 column.

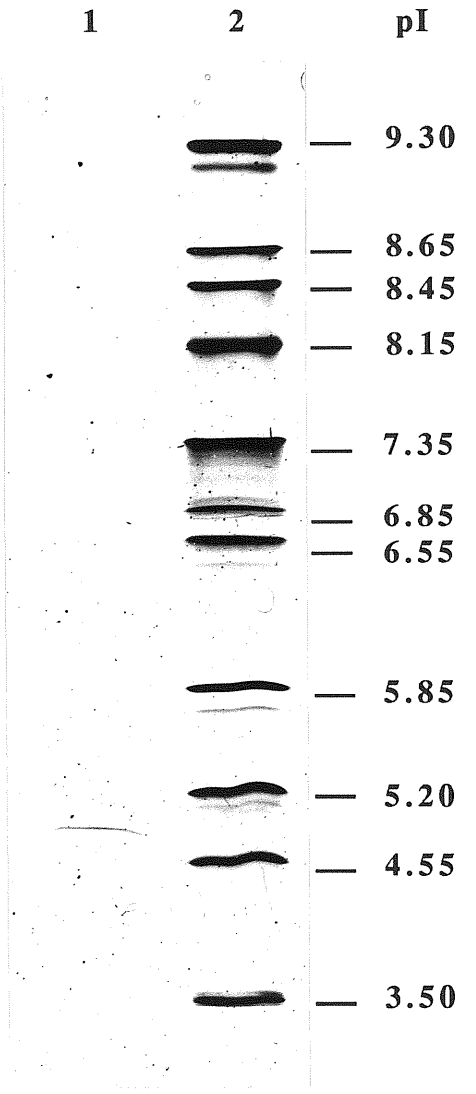


Fig. 18. Analytical isoelectric focusing assay of the peak fraction from Sephacryl HR200 column on a polyacrylamide gel. Lanes: 1, purified recombinant ferulate decarboxylase; 2, pI markers.

The hydropathy profile (Fig. 19) determined for the predicted amino acid sequence indicates the ferulate decarboxylase to be a highly hydrophylic and probably cytoplasmatic 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 proteins synthesized from *B. pumilus* and *E. coli* are very similar. However, the ferulate decarboxylase expressed in *E. coli* seems relatively unstable, since large part of the activity was lost during purification.

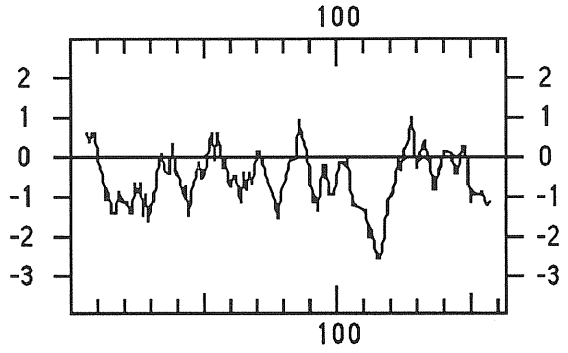


Fig. 19. Hydropathy profile (Kyte-Doolittle) of the recombinant ferulate decarboxylase

3.2.5 *B. pumilus fdc* related DNA sequences in *B. licheniformis* and *B. subtilis*.

Since ferulic and *p*-coumaric acid decarboxylase has been isolated in our laboratory from *B. licheniformis* LMG 6933 and *B. subtilis* 1012, we investigated the homology among the genes coding for this activity.

The *B. pumilus fdc* gene was amplified by PCR with oligonucleotides corresponding to sequences at 5' (A1: CTATTTTCGTCCAGCTCTGATCT) and 3' (A2: TGGATCAATTCGTTGGACTTC) ends of the gene. This PCR product was labelled and used as a probe to perform a Southern blot analysis of *B. licheniformis* and *B. subtilis*

DNA, totally digested with *Hin*dIII. Under conditions of low stringency a single discrete band of hybridization of about 5 Kb and a higher band, around 8.5 Kb, were seen with digested *B. licheniformis* and *B. subtilis* DNA, respectively (Fig. 20). This result confirms a certain grade of homology among these genes, as suggested by the comparison of the amino-terminal portion of the proteins determined by stepwise Edman degradation.

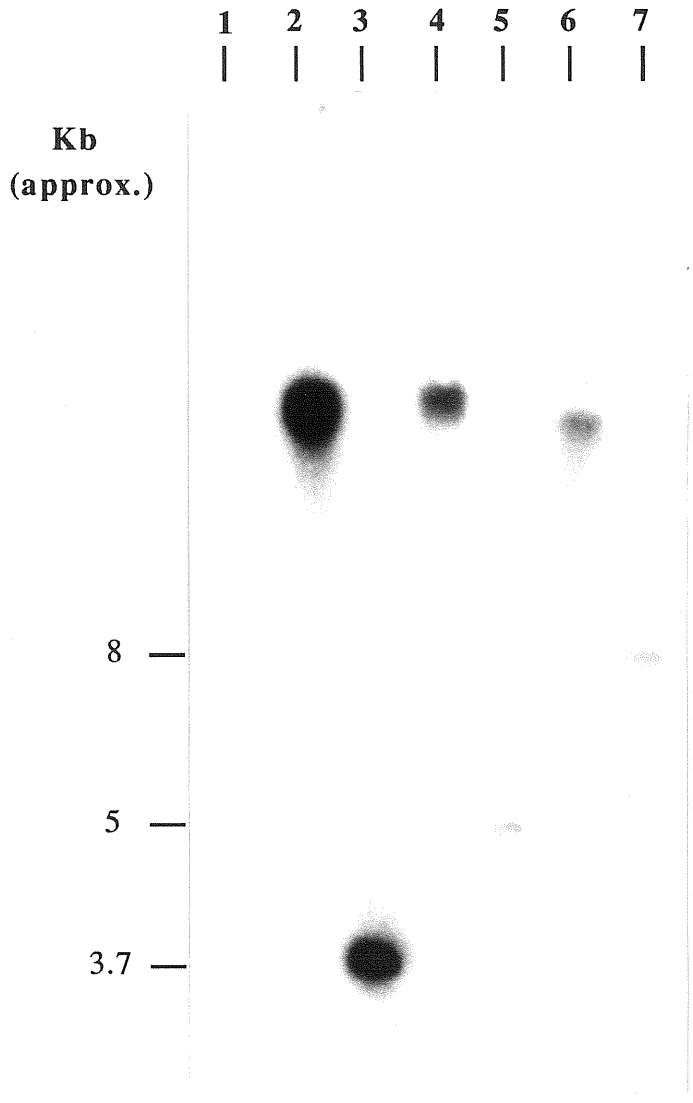


Fig. 20. Southern blot analysis of *B. pumilus*, *B. licheniformis* and *B. subtilis* total DNA using as a probe *B. pumilus fdc*. (**1**) *E. coli* DH5α total DNA, (**2**) *B. pumilus* undigested DNA, (**3**) *B. pumilus* DNA digested with *Hin*dIII, (**4**) *B. licheniformis* undigested DNA, (**5**) *B. licheniformis* DNA digested with *Hin*dIII, (**6**) *B. subtilis* undigested DNA, (**7**) *B. subtilis* DNA digested with *Hin*dIII

Section 3

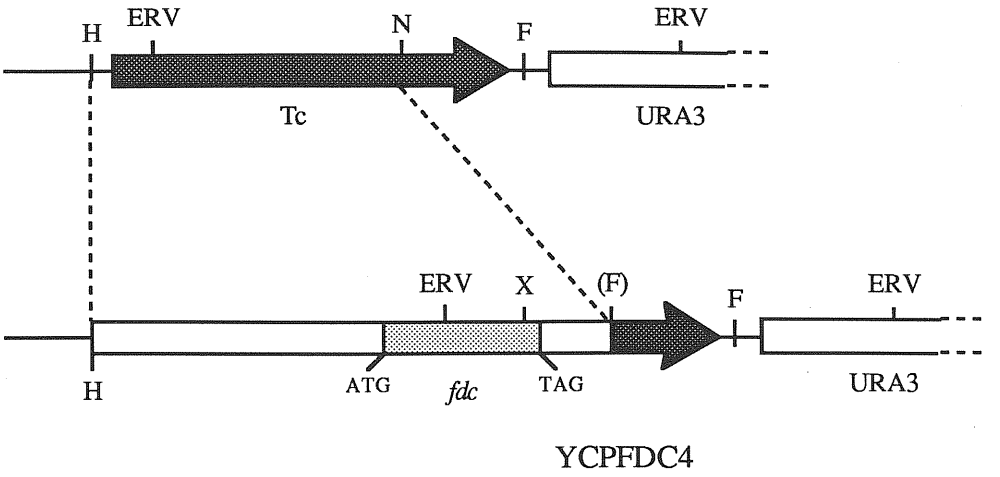
3.3 Expression of the *fdc* gene in yeast

3.3.1 Introduction

In brewing ferulic and p-coumaric acids are released when malted barley is extracted with warm water. These substrates are decarboxylated to 4-vinylguaiacol and 4-vinylphenol respectively by S. cerevisiae and this activity is associated with the production of a phenolic off-flavour in beer. The ability of Saccharomyces cerevisiae to carry out the phenylacrylic acid decarboxylation (PAD) reaction has been assigned to a single non-essential gene PAD1 (Goodey and Tubb, 1982). PAD1 was cloned by phenotypic complementation and its sequence was determined (Clausen et al., 1994). A pad1 S. cerevisiae strain, which lacks PAD activity was constructed by gene disruption in our laboratory. The ability of the B. pumilus fdc gene to complement this S. cerevisiae pad1 mutant was studied.

3.3.2 Cloning and expression of the *B. pumilus* ferulate decarboxylase in the yeast *Saccharomyces cerevisiae.*

To study the expression of the *fdc* gene from *B. pumilus* in *S. cerevisiae* the constructions shown in Fig. 21 were made using the yeast-*E. coli*-shuttle vectors Ycp50 and pVT100-U. A 1521 bp *Hin*dIII-*Fsp*I *B. pumilus* DNA fragment isolated from the pUCFDC plasmid was ligated into Ycp50 digested with *Hin*dIII and *Nru*I. The *B. pumilus* DNA fragment contained the complete *fdc* ORF and non coding flanking sequences, respectively 816 nucleotides upstream from the ATG starting codon and 219 nucleotides downstream from the TAG stop codon. The *Hin*dIII-*Nru*I fragment deleted from the Ycp50 vector contained part of the *tet*R gene and it was substituted with the *B. pumilus fdc* gene. The plasmid construction was inserted into *E. coli* DH5α and ampicillin resistant and tetracycline



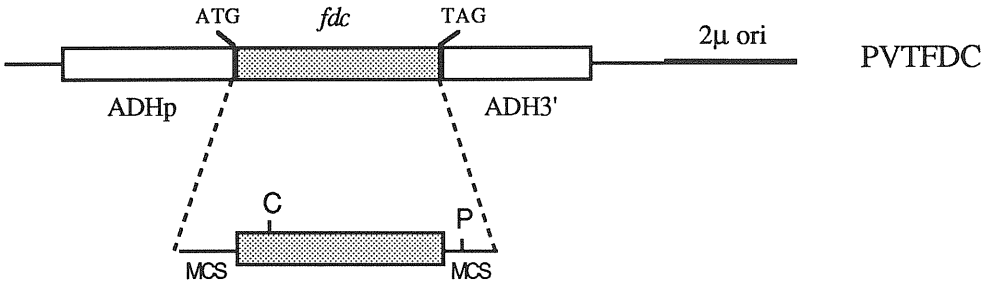


Fig. 21. **(A)** Cloning into YCp50 of a *Hin*dIII-*Fsp*I *B. pumilus* DNA fragment containing the *fdc* coding sequence (shaded bar) and the non-coding flanking regions (white bar).
(B) Cloning of the *fdc* coding region into the multicloning site of pVT100 downstream the *ADH1* promoter and upstream the *ADH1* terminator. Ap, ampicillin; Tc, tetracycline; C, *Cla*I; ERV, *Eco*RV; F, *Fsp*I; H, *Hin*dIII; N, *Nru*I; P, *Pst*I; X, *Xba*I.

sensitive colonies were collected. Some of these colonies were checked by restriction enzyme analysis with *Xba*I and *Eco*RV and those which showed linearization of their plasmid by *Xba*I restriction and a fragment of about 1.2 Kb after digestion with *Eco*RV, confirming the correct insertion of the *B. pumilus* DNA fragment, were tested for their ability to decarboxylate ferulic acid *in vivo*. Our results showed that all of them had acquired the ferulate decarboxylation activity. Plasmidic DNA (YCPFDC) extracted from one of these positive clones was transformed into a *S. cerevisiae* YPH250 strain which lacks the ferulate decarboxylase activity since the *PAD1* gene has been disrupted. To determine if the *B. pumilus fdc* gene was able to complement the *pad1* mutation, Ura3+ transformants containing the *fdc* gene were assayed for their ability to decarboxylate ferulic acid. The supernatant of a 4 days culture of some of these mutants in soybean-meal glucose containing 0.5 g of ferulic acid per liter was analyzed by HPLC (Fig. 22). No complementation was observed.

Since putatively heterologous (non yeast) regulatory sequences may not work in yeast, the coding region of the *fdc* gene, amplified by PCR, was inserted into the unique *Pvu*II site of the yeast expression vector pVT100. This gene was inserted under the control of the yeast alcohol dehydrogenase (*ADH1*) promoter region and upstream from the transcription termination of the *ADH1* gene to produce the plasmid PVTFDC. This construct was inserted into *E. coli* DH5α and clones containing the *fdc* gene were identified by Southern blot analysis. The right orientation of the gene with respect to the *ADH1* promoter was checked by double digestion with *Cla*I and *Pst*I restriction enzymes and by sequencing, using the primers indicated by Vernet *et al.*, 1987. The sequence also confirmed that no mutations were introduced in the PCR product of the *fdc* ORF, which was cloned in pVT100. Transformation of the *pad1* mutant yeast strain YPH250 with PVTFDC resulted in the expression of the bacterial *fdc* gene as demonstrated by a strong decarboxylase activity detected by HPLC analysis of the supernatant of 4 days cultures of transformants grown in soybean-meal glucose containing 0.5 g of ferulic acid per liter (Fig. 23).

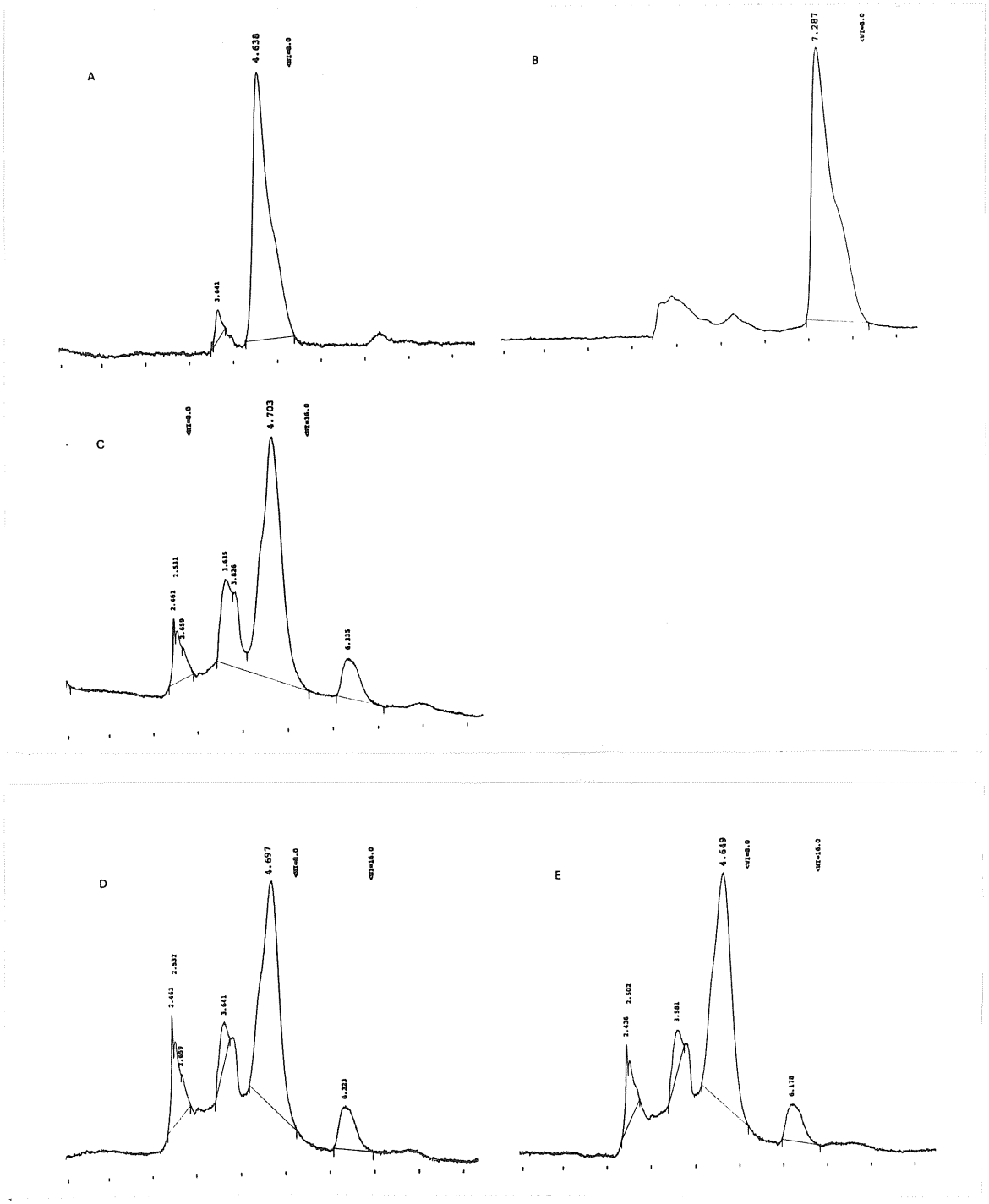


Fig. 22. HPLC retention time of **(A)** ferulic acid 0.5 µg ml^{-1} standard; **(B)** 4-vinylguaiacol 0.5 µg ml^{-1} standard; **(C)** soybean-meal medium plus 0.5 µg ml^{-1} ferulic acid; **(D)** soybean-meal medium plus 0.5 µg ml^{-1} ferulic acid after an overnight growth of *pad1* disruptant *S. cerevisiae* YPH250; **(E)** soybean-meal medium plus 0.5 µg ml^{-1} ferulic acid after an overnight growth of *pad1* disruptant *S. cerevisiae* YPH250 bearing YCPFDC. λ=260 nm and flow rate = 0.5 ml min^{-1} (Varian LC Star Program).

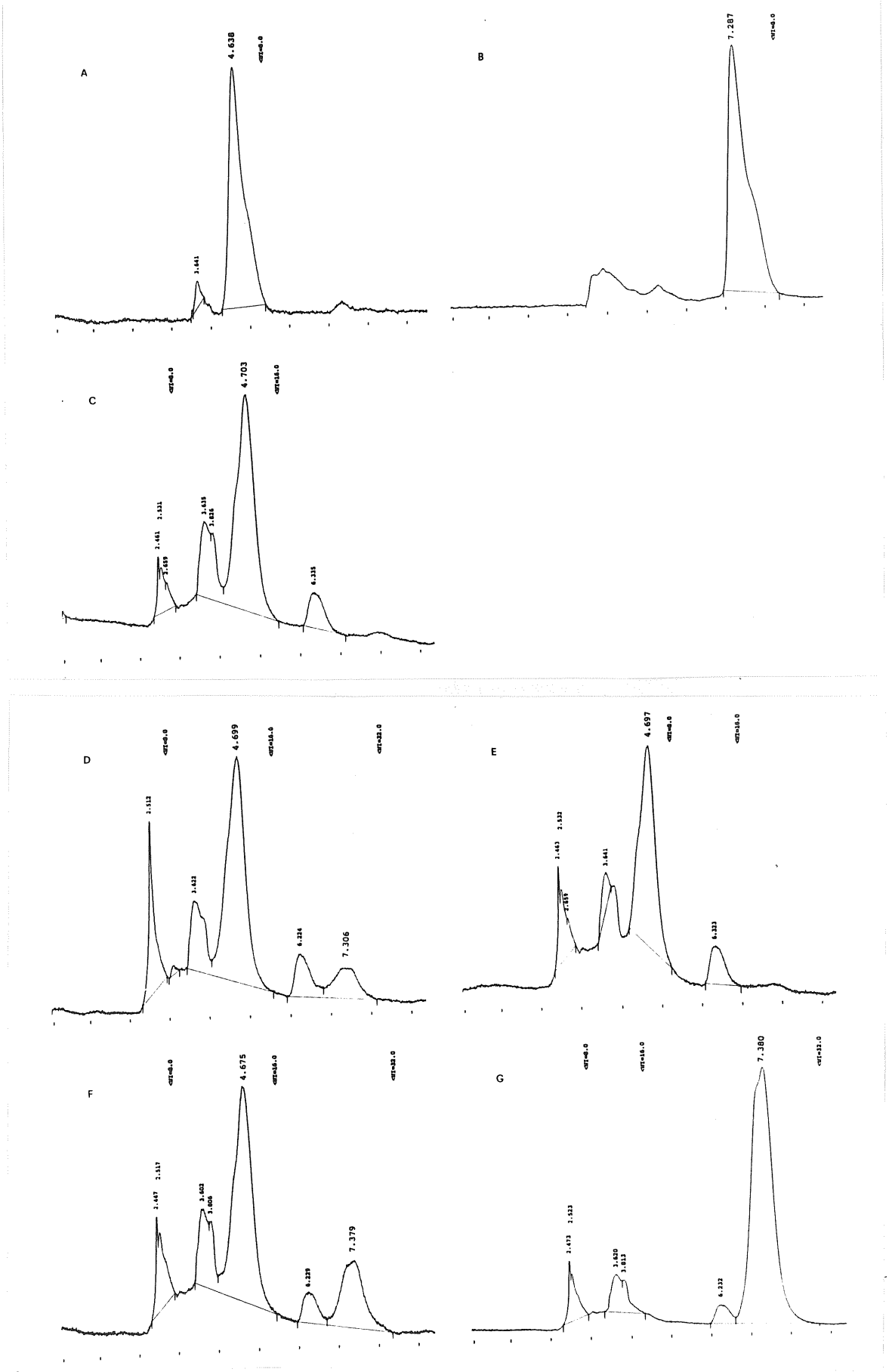

A
4.638
3.441
B
7.287
C
4.703
2.461
2.521
3.618
3.826
6.335
D
4.699
2.512
3.422
6.224
7.306
E
4.697
2.332
2.463
3.441
6.323
F
4.675
2.447
2.517
3.602
3.806
6.329
7.379
G
7.380
2.473
2.522
3.420
3.813
6.332

Fig. 23.

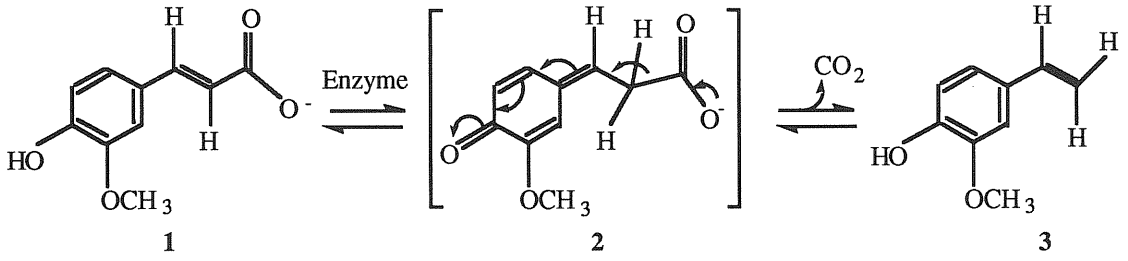
Fig. 23. HPLC retention time of (**A**) ferulic acid 0.5 μg ml^{-1} standard; (**B**) 4-vinylguaiacol 0.5 μg ml^{-1} standard, (**C**) soybean-meal medium plus 0.5 μg ml^{-1} ferulic acid; (**D**) soybean-meal medium plus 0.5 μg ml^{-1} ferulic acid after an overnight growth of wild type *S. cerevisiae* YPH250; (**E**) soybean-meal medium plus 0.5 μg ml^{-1} ferulic acid after an overnight growth of *pad1* disruptant *S. cerevisiae* YPH250; (**F**) soybean-meal medium plus 0.5 μg ml^{-1} ferulic acid after an overnight growth of *pad1* disruptant *S. cerevisiae* YPH250 bearing *PAD1* in pVT100; (**G**) soybean-meal medium plus 0.5 μg ml^{-1} ferulic acid after an overnight growth of *pad1* disruptant *S. cerevisiae* YPH250 bearing *fdc* in pVT100. λ=260 nm and flow rate = 0.5 ml min^{-1}(Varian LC Star Program).

4. DISCUSSION

The rumen is one of the environments where the lignocellulose of plant cell walls is potentially degraded, owing to the presence of bacteria and fungi which utilize the polysaccharide components, lignin and lignin monomeric compounds. Lignocellulose degradation, which results in its mineralization, is a very complex process involving many enzymatic reactions and it is widely assumed that ruminal degradation of plant cell walls is a combined action of several microorganisms highly specialized. In this study we focused our attention on the microbial catabolism of phenolic monomers released by cell wall degradation. We analyzed the degradation abilities of the components of a microbial consortium from rumen fluid, which was previously demonstrated, in our laboratory, to function efficiently in the anaerobic degradation of some lignin related aromatic compounds and in the polymeric dye poly S. The decolorization assay of the poly S (Field *et al.*, 1992) might be a further indication of the lignin degradation abilities of rumen microorganisms because of the presence in this dye of bonds which are structural in the lignin. As a result of this screening program I isolated and characterized some bacteria that in the rumen microbial consortium could be involved in the anaerobic utilization of ferulic acid, *p*-coumaric acid, vanillic acid, *p*-hydroxybenzoic acid and sinapic acid. Perhaps 2 days incubation was too short to detect also the modification of veratrylaldehyde, *trans*-cinnamic acid, vanillin, veratric acid, syringaldehyde and syringic acid. Moreover, the lack of complete mineralization of these aromatic compounds is a confirmation that it is usually the product of a microbial co-operation. However, further investigation of the biotransformation products is necessary, while longer periods of incubation could give more information. It could also be interesting to compare these activities to those of bacteria washed from the rumen particulate, strictly associated to the substrate. Degradation activity was tested in anaerobiosis since it is usually assumed that the rumen provides a highly anaerobic environment, although a significant entry of O_2 must occur. Most rumen microorganisms grow and function without O_2, however rumen contains facultatively anaerobic bacteria that

can tolerate O_2 or even utilize it. It has been shown that O_2 is detectable in the rumen fluid after feeding of the animal (Scott *et al.*, 1983). Rumen compartmentalization could justify the presence in the rumen of strictly anaerobic as well as facultative anaerobic and strictly aerobic organisms. The strains that we isolated from rumen fluid were prevalently aerotolerant. Only three facultative anaerobes showed activity toward hydroxycinnamic as well as benzoic compounds, all the other bacterial strains were able to modify preferentially the cinnamic compounds and lack activity toward the benzoic ones. Modification of ferulic and *p*-coumaric acids has been observed to be widely spread among rumen bacteria and also modification of sinapic acid could be often detected in association or independently from the modification of ferulic and *p*-coumaric acid. The pattern of modification of these hydroxycinnamic compounds was very similar, suggesting that each substrate was modified in the same way by all strains active upon them. Moreover, the transformation of these three phenolic lignin monomers has been detected among all the collection *Bacillus* strains tested, which are known to contribute positively to the complex microbial interactions affecting plant cell wall breakdown and utilization in the rumen ecosystem. Particularly, we focused our attention on the biotransformation of *p*-coumaric and ferulic acid by a *B. pumilus* strain, repeatedly isolated from rumen fluid in our laboratory. Ferulic acid is completely degraded under anaerobic conditions and in anaerobiosis by different microorganisms and possible pathways of ferulate metabolism have been proposed (Healy *et al.*, 1980; Grbic-Galic, 1985; Grbic-Galic and Young, 1985; Grbic-Galic, 1986; Nazareth and Mavinkurve, 1986). However, we did not detect mineralization of ferulic acid and *p*-coumaric acid, but a modification which has been investigated in *B. pumilus* and revealed to be a decarboxylation. Ferulic acid decarboxylation by *B. pumilus* DSM 361 has been demonstrated by Arfman and Abraham, 1989. These and other authors showed the same activity in several other microorganisms (Nazareth and Mavinkurve, 1986; Huang *et al.*, 1993a; Huang *et al.*, 1993b). However we further investigated this activity in the rumen isolated *B. pumilus* and demonstrated its inducibility by the substrate, in contrast with the constitutivity of the ferulate decarboxylase produced by *P. fluorescens* UI-670 (Huang *et al.*, 1994). The enzyme of *B. pumilus* was isolated and characterized in our laboratory. It

revealed to be a homodimer consisting of about 23 KDa subunits, as already reported for *P. fluorescens* enzyme. Significant biochemical differences were observed between the two purified enzymes, although a comparison of the respective N-terminal amino acid sequences showed good homology. The *B. pumilus* enzyme was able to work in anaerobiosis as well as in aerobiosis, which is in accordance with the distribution of this activity among strictly anaerobic as well as facultative or obligatory aerobic microorganisms. In fact the modification of the spectra of ferulic and *p*-coumaric acid observed, after incubation with some anaerobic rumen bacteria in anaerobiosis and facultative anaerobic Bacilli in aerobiosis, matched the modification of these two compounds by the ferulate decarboxylase of the strictly aerobic *B. pumilus*. The mechanism for the non-oxidative decarboxylation of ferulic acid (1) to 4-hydroxy-3-methoxystyrene (3) involves the conversion of ferulic acid into a quinoid tautomer (2) (a vinylogous ß-keto acid) that should spontaneously decarboxylate.



This mechanism, analogous to that proposed by Huang and co-workers (1993) for the ferulate decarboxylase of *P. fluorescens*, was confirmed by us to be valid also for the *B. pumilus* ferulate decarboxylase (data not shown). Biotransformation of ferulic acid in the presence of deuterium oxide gave stereospecific incorporation of deuterium into the styrene as determined by NMR analysis suggesting a stereospecific enzymatic reaction.

Bacillus species have already been studied for their ability to degrade a large variety of low molecular weight benzenoid molecules and three different pathways of degradation of 4-hydroxybenzoate by this species have been investigated (Crawford, 1975; Crawford, 1976). Decarboxylation of vanillate to guaiacol has been reported (Crawford and Perkins-

Olson, 1978). *B. pumilus* is not able to modify vanillic acid and its decarboxylase activity seems to have a high substrate specificity. Ability to modify sinapic acid has been detected among all *Bacillus* tested and seemed to be strictly associated to the decarboxylation of the other two hydroxycinnamic acids analyzed, while they did not degrade *trans*-cinnamic acid, which lacks the hydroxy group in para. It was very difficult to demonstrate that the decarboxylation of ferulic and *p*-coumaric acid is independent from the modification of sinapic acid, because its product was not commercially available. *B. pumilus* is known to be able to hydrolyze xylan (Panbangreb *et al.*, 1984, Panbangreb *et al.*, 1983) by sequential reactions; the first is the conversion of xylan to oligosaccharides by extracellular endo-xylanases followed by hydrolysis to xylose by intracellular ß-xylosidases. Since hemicellulose bears characteristic substituents on the main polymer chain, (O-acetyl groups, arabinose, uronic acid and cinnamic acid based esters) which could limit the rate of degradation of hemicellulose, substituent hydrolysing activities are required for its complete degradation. *B. pumilus* produces an esterase induced by corn stalk which is active toward the colorimetric test substrate naphtylacetate, and is going to be purified in our laboratory. Its role in the cell wall degradation and its co-operativity with the xylan-hydrolysing enzymes, already demonstrated *in vitro* for some acetylesterases (Biely *et al.*, 1986), remain to be investigated. Since rumen bacteria able to digest cellulose and hemicellulose are strictly associated with plant material undergoing degradation, they are likely to encounter localized high concentrations of released phenolic acids. *B. pumilus* does not utilize ferulic acid and *p*-coumaric acid as sole carbon source, but its decarboxylase activity could be a detoxification mechanism, if the respective products, 4-vinylguaiacol and 4-vinylphenol, were better tolerated by this bacterium. However, ferulic acid and 4-vinylguaiacol seem to equally inhibit the growth of *B. pumilus* when their concentration in the medium is higher than 5 mM (data not shown) and we could not demonstrate that ferulic acid interferes with other bacterial enzymatic activities in the rumen. Alternately, this reaction could be just a step of a slow complete degradation pathway that we could not detect in a seven days interval under the conditions tested.

The gene encoding the ferulate and *p*-coumarate decarboxylase in *B. pumilus* PS213 has been identified. The *fdc* gene was cloned and expressed in the heterologous host *E. coli* DH5α, that normally lacks this decarboxylase activity. We established the complete nucleotide sequence of the *fdc* gene (486 bp) and its flanking regions. Upstream the initiation codon, a potential promoter with consensus sequences very similar to those found in *E. coli* was detected. It seemed to be functional in *E. coli*, although additional studies are necessary to exclude the possibility that the gene was expressed under the control of the LacZ promoter localized more distantly in the plasmid. No homology was found with the promoter regions of the *B. pumilus xynA* and *xynB* genes (Moriyama *et al.*, 1987).

The inducibility of the *fdc* gene was lost in the heterologous host suggesting that a possible regulatory element lacks on the DNA fragment cloned in *E. coli* or, most likely , *E. coli* does not possess the genetic information for the regulation of this gene. In fact, when the total protein pattern from a *B. pumilus* culture induced with ferulic acid was compared to that of a non induced culture two new bands clearly appeared. One of these corresponded to the ferulate decarboxylase enzyme and another was supposed to be a regulator. The N-terminal sequence of this "possible" regulator protein has been defined, but its coding DNA sequence remain to be identified. There is no homology with the 5' end of the unknown ORF observed on the complementary DNA strand in the opposite orientation with respect to the *fdc* gene. The *fdc* gene conferred to *E. coli* DH5α the ability to degrade ferulic and *p*-coumaric acid. On the other hand, *E. coli* DH5α and the transformed strain containing the *fdc* gene were able to modify the UV spectrum of sinapic acid in the same way. Although the product of this modification was difficult to identify, it was clear that the ferulate decarboxylase was not responsible for this modification.

The ferulate decarboxylase (Fdc) predicted amino acid sequence did not show similarity to any other protein currently in sequence databases. The recombinant protein was purified from *E. coli* and characterized. Biochemical studies showed that it corresponded to the protein expressed in *B. pumilus*. The molecular mass (experimentally determined as 21,500 Dal) is near to the 23,000 Dal of the native *B. pumilus* protein and both of them are assembled in a functional homodimer. Moreover, the deduced amino acid sequence at the 5'

end of the *fdc* gene matched the N-terminal amino acid sequence of the of the *B. pumilus* enzyme confirming this identity.

The isoelectric point of the ferulate and *p*-coumarate decarboxylase isolated from *B. pumilus* determined by means of isoelectric focusing was 4.6. The isoelectric focusing protein pattern showed a major band at pH 4.6 and two faint bands at slightly higher pH, in accordance to the HPLC analysis of the same protein, which showed a major peak together with two smaller peaks eluted earlier. This suggested the existence of three isoenzymes and consequently three isogenes, condition which is not very frequent among bacteria. The isoelectric focusing of the recombinant decarboxylase showed a major band at pH 4.8 and two very faint bands (undetected by HPLC) at lower pH. One of them corresponded to the isoelectric point 4.6 of the native protein. Since the only codon usage available for *Bacillus* species (*B. subtilis*) is different from that of *E. coli* (Ogasawara, 1985), it could be inferred that the observed isoforms were due to amino acid misincorporation (Scorer *et al.*, 1991) or to chemical modification occurred during the purification steps. A 24 KDa and a 20.6 KDa ferulate decarboxylase were purified from *B. licheniformis* LMG 6933 and *B. subtilis* LMG 7135 respectively. This activity is inducible in both species and the biochemical properties of the relative two proteins are similar to those of the *B. pumilus* enzyme. Their N-terminal amino acid sequences obtained by stepwise Edman degradation are comparable.

B. licheniformis LMG 6933	M N Q D V K E F V G S H M I Y T Y E N
B. subtilis LMG 7135	M E N F I G S H M I Y T Y E N G W E Y E I Y I Y N V
B. pumilus PS213	M D Q F V G L H M I Y T Y E N G W

Southern blot hybridization under low-stringency conditions, using *fdc* as probe and screening genomic DNA of both *Bacillus* species confirmed a certain degree of identity among these genes. The coding sequence of the ferulate decarboxylase of *B. licheniformis* LMG 6933, isolated by reverse genetics, is being sequenced.

All these results agree with the ubiquity of the ferulate and *p*-coumarate decarboxylase activity among *Bacillus* spp., nevertheless current data are not strong enough to conclusively justify a wide distribution of this activity among Gram-positive and Gram-negative, as well as aerobic and anaerobic microorganisms. Our investigation was limited to

bacteria that are present in the soil and in the rumen, environments where high concentrations of ferulic and *p*-coumaric acid can be present. In this case the possible explanation of the presence of this enzyme is the detoxification role of the ferulate and *p*-coumarate decarboxylase, however, minor toxicity of guaiacol derivatives of the two hydroxycinnamic compounds remains to be clearly demonstrated. On the other side we can not exclude that the decarboxylation of ferulic and *p*-coumaric acid is part of a secondary metabolism that remains to be understood. For example, several organic acid decarboxylases have been recently shown to be primary sodium pumps. These decarboxylases are capable of converting the chemical energy of the high exergonic decarboxylation reactions into electrochemical Na^+ gradients. However, Na^+ transport is not the only function of physiological importance of the transport decarboxylases. The decarboxylation of the organic acid oxalacetate and methylmalonyl-CoA are per se indispensable steps in the anaerobic degradation of citrate and lactate (Rosen, 1986). One of our current goals is to create a *fdc* mutant *Bacillus subtilis* strain to investigate the role of this gene in the cell. Since repeated attempts to transform *B. pumilus* PS213 failed, we focused our attention on the well known and genetically amenable *B. subtilis*, which is known to decarboxylate ferulic and *p*-coumaric acid. As previously shown, it should have a ferulate decarboxylase gene which shares a certain homology with *B. pumilus fdc* gene. We have inactivated *in vitro* the *B. pumilus fdc* gene by insertion of a kanamycin (Km^R) cassette and we are trying to insertionally inactivate the *fdc* locus in *B. subtilis* genome by a marker exchange procedure *in vivo* between the target plasmid carrying the gene with the Km fragment and the corresponding region of the genome. This mutant should give further information on the phenotype derived by this gene.

The single dominant *PAD1* gene, which codes for a phenylacrylic acid decarboxylase in *S. cerevisiae* has been identified ad sequenced by Clausen and co-workers, 1993. *PAD1* locus was mapped 140 Kb from the left end of chromosome IV. This gene confers the ability to decarboxylate phenylacrylic acids, among which ferulic and *p*-coumaric acids, to produce vinyl derivatives. The presence of these latter compounds in beer produces an undesirable off-flavour, therefore the elimination of the Pad character is of wide significance

to the brewing industry and a *S. cerevisiae* Pad$^-$ mutant strain was constructed in our laboratory. On the other hand, in Gewürztraminer wine this aroma is regarded as characteristic of the wine (Grando *et al.*, 1993). The predicted 242 amino acid Pad polypeptide does not show significant homology to the *fdc* gene but it is 48,6% identical to the unknown product of the *dedF* within the *purF* operon of *E. coli*, involved in *de novo* purine biosynthesis (Tso *et al.*, 1982). The *S. cerevisiae* Pad$^-$ mutant strain was used to study the expression of the prokaryotic ferulate and *p*-coumarate decarboxylases in yeast and to investigate its ability to complement the *pad* mutation. The *fdc* gene was initially cloned under the control of its regulatory regions to study the function of these heterologous signal sequences in yeast. It has been reported that the bacterial promoter or promoter-like sequences of the *B. amyloliquefaciens* α-amylase-encoding gene (*AMY*) (Pretorious *et al.*, 1988), the *E. coli* chloramphenicol acetyltransferase-encoding gene (*cat*) (Cohen *et al.*, 1983) and the aminoglycoside phosphotransferase-3'(I)-encoding gene (*kan*) (Jimenez and Davies, 1980) can fortuitously direct gene expression in *S. cerevisiae*. *The* expression of prokaryotic DNA in yeast involves the recognition of their heterologous regulatory signals. *B. pumilus* DNA was inserted in a *E. coli*/yeast shuttle vector where large part of the *tet* promoter (Harley *et al.*, 1988) and *tet* coding sequences had been deleted. The *B. pumilus* promoter-like sequence did not function in yeast but the same DNA worked efficiently in *E. coli* DH5α where it was amplified. This supported the hypothesis of an active function of the Gram-positive *B. pumilus* promoter in *E. coli*. However, when the *fdc* bacterial gene was inserted between a yeast promoter and a yeast transcription terminator, expression of the gene was obtained and the bacterial enzyme was more active toward the hydroxycinnamic compounds, ferulic and p-coumaric, than the yeast phenylacrylic acid decarboxylase.

In conclusion the role of *B. pumilus* decarboxylase in the lignocellulose degradation remains to be clarified and more investigation is necessary to exploit the cooperative function of this microorganism in the rumen consortium. The knowledge of this function is necessary to explore a biotechnological application of this microorganism to improve the digestion of feedstuff by ruminants. However the microbial biocatalysis described in this work could have an immediate application in the commercial production of guaiacol derivatives.

Vinylphenol derivatives are reported as being constituents in the flavours of soya sauce and blueberries, while 4-vinylguaiacol is used by the fragrance and perfume industry. Today, skilled flavourists and perfumers must respond quickly to customer demands for new formula and frequently depend on modern biotechnology to provide many of the raw materials used to produce flavours and fragrances. An understanding of the basic microbial processes associated with production of flavour and fragrance chemicals is essential for the development of these unique fermentation processes.

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