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IDENTIFICATION OF BACTERIAL ENZYMES ACTIVE ON MONOMERIC LIGNIN AROMATIC COMPOUNDS

Thesis submitted for the Degree of
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TRIESTE

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1. INTRODUCTION

1.1 Structure and biosynthesis of lignin

Lignin occurs in cell walls of vascular plants. It is generally distributed as a matrix component with hemicellulose in the spaces between cellulose-microfibrils in primary and secondary cell walls. Lignin is also found in the middle lamellae responsible for the connection of the cells. It gives rigidity to the plant cell wall which helps the cell to resist harsh environmental conditions. Lignin also decreases water permeation across cell walls in conducting xylem tissues, thus preventing water leakage from cell walls and playing an important role in the internal transport of water, nutrients and metabolites in the plant.

Lignin is an amorphous aromatic polymer, resistant to hydrolysis, which comprises some 20 to 30% dry weight of wood. It is composed of highly branched polymeric molecules consisting of phenyl-propane-based monomeric units linked together by different types of bonds, including alkyl-aryl, alkyl-alkyl and aryl-aryl ether bonds (Kirk *et al.*, 1980).

Lignin is synthesized in nature starting from the cinnamyl alcohols or monolignols, p-coumaryl, coniferyl and sinapyl alcohols. In lignin these monomers are referred to as p-hydroxyphenyl, guaiacyl and syringyl residues. Their hydroxyl group is oxidized by peroxidase or laccase enzymes thus yielding free radical species that couple in a non enzymatic and random fashion to form dilignols, oligomeric intermediates and finally lignin macromolecules (Fig. 1).
Lignins from most gymnosperm 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.

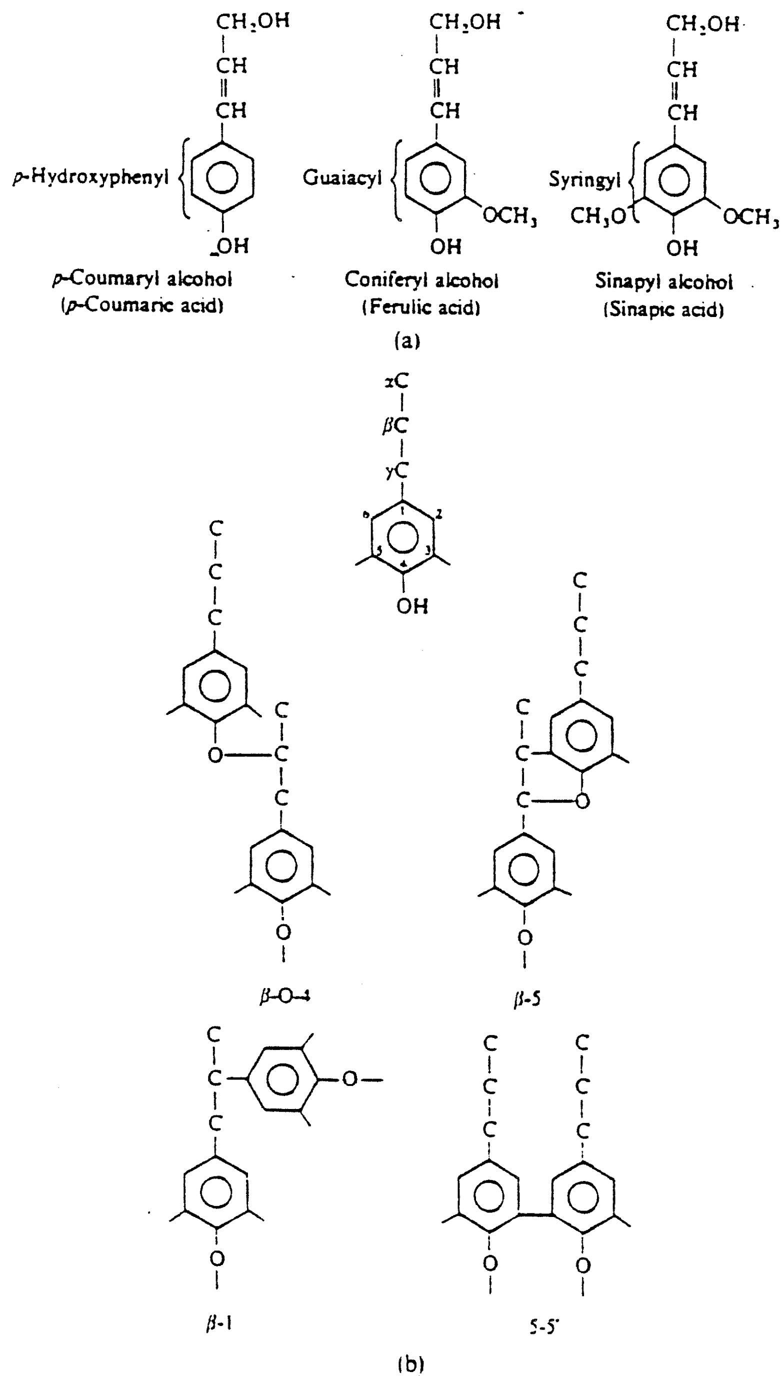


FIG.1 (a) Structure of the major alcohol precursors of lignin with the equivalent phenolic acids shown in parenthesis, and (b) the four major aryl ether and carbon-to-carbon linkages found after polymerisation of lignin precursors; dimers are shown in skeletal form only (Hobson P. N.,1988).

1.2 Microbiology of lignin degradation

Nature has developed an efficient mechanism for the modification and degradation of lignin. The biological degradation of lignin is one of the most important parts of the biospheric carbon-oxygen cycle. Efforts to find and isolate microorganisms with lignin decomposing activity have been fruitful and the range of organisms known to attack lignin is expanding. It is likely that the complete mineralization of lignin in natural environments requires the concerted action of different types of microorganisms (Vicuña, 1993). Most of the attention has been concentrated on ligninolytic fungi since their ability to attack various polymers present in wood is well documented. White-rot fungi (basidiomycetes and few ascomycetes) attack interlignols bonds, cleave aromatic rings, cause aromatic hydroxylation and demethylation of methoxyl groups and brown-rot fungi (basidiomycetes) are responsible for aromatic hydroxylation, demethylation and limited side-chain oxidation (Kirk and Farrell, 1987; Kirk *et al.*, 1981).

Ligninolytic bacteria have been much less studied: lignin mineralization levels observed with actinomycetes are much lower than those of white-rot fungi, although they can reach values close to 15%. In any case lignin is modified by the action of these filamentous bacteria, the main reactions involved being demethylation and oxidations (Vicuña *et al.*, 1993). Non-filamentous bacteria are involved in the decomposition of low molecular weight aromatic compounds released from the polymeric substrate by the primary action of fungi. However three gram-negative aerobic bacteria, identified as *Pseudomonas*, *Xanthomonas* and *Acinetobacter*, were reported to be capable of degrading milled wood lignins at rates of 4 to 20% within a 7 days period (Odier *et al.*, 1983). Several *Pseudomonas* strains have been isolated able to proliferate in synthetic media containing dilignols as the only source of carbon and energy. This implies that bacteria possess intracellular enzymes that cleave lignin specific linkages (Vicuña *et al.*, 1993).

1.3 Anaerobic degradation of monoaromatic lignin derivatives

Since polymeric lignin is composed of aromatic subunits, any investigation of its microbial degradation requires an understanding of the mechanism by which aromatic rings are metabolized. Until recently, catabolism of aromatic compounds was thought to be a strictly aerobic process. Nowadays there are several evidences of anaerobic degradation of aromatic compounds.

Anaerobic metabolism of aromatic monomers is known to occur during anaerobic photometabolism in several species of purple, non sulfur bacteria (*Rhodopseudomonas*, *Rhodocyclus*, *Rhodospirillum*), under nitrate-reducing conditions as demonstrated by investigations in a *Pseudomonas* sp. and an aromatic-degrading *Moraxella* sp., under sulfate reducing conditions, in a microbial consortium, where fermentation is often coupled with methanogenesis and by pure culture of fermentative bacteria (Calberg, 1988).

Demethoxylation, decarboxylation, dehydrogenation, demethylation are all possible under strictly anaerobic conditions. In the absence of molecular oxygen all systems that degrade benzenoid structures work through reduction of the ring followed by cleavage (Evans and Fuchs, 1988).

1.4 Degradation of polymeric lignin and aromatic monomers in the rumen

Phenylpropionic acid in rumen fluid was noted to be significantly greater that could be accounted for by aminoacid utilization of the microflora (Hungate and Stack, 1982). Initially there was some concern in postulating that some phenylpropionate may have come from anaerobic breakdown of lignin since it was considered "indigestible" and fermentation of lignin had not been established in ruminants. However, recent experimental data showed that lignin is in some way solubilized or transformed during its residence in the rumen (Susmel and Stefanon, 1993). A 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 degrades different lignocellulosic substrates and lignin monomers in vitro (Akin and Benner, 1988; Akin and Rigsby, 1987; Borneman *et al.*, 1989; Degrassi *et al.*, 1993). In addition the formation of soluble lignin carbohydrate complexes in the rumen due to microbial activity was reported (Gaillard, 1975).

The isolation of molecular complexes which contain both carbohydrate and lignin is also a presumptive evidence for the existence of a chemical association between them. This association is very important since lignin is the major plant cell wall component leading to decreased structural carbohydrate utilization by ruminal bacteria and delignification substantially enhances cell wall digestion of lignified tissues (Chesson, 1981). The polysaccharide-lignin cross linkages are glycosylation of lignin hydroxyls by the polysaccharides, direct ether and ester cross-linkages, while the presence of hydroxycinnamic acids esterified to wall polysaccharides and the detection of ether linked hydroxycinnamic acids on lignin have suggested the existence of a cinnamic acid bridge (Conchie *et al.*, 1987). Therefore, in plant cell wall, two phenolic components can be distinguished, including polymers (core lignin) and low molecular weight monomers that bind lignins to the hemicellulose and cellulose (non-core lignin). Non-core lignin has a high content of ferulic acid and *p*-coumaric acid, mainly in their *trans* configuration, (Smith and Hartley, 1983; Mueller-Harvey and Hartley, 1986) (Fig.2). It is particularly abundant in Graminaceous plants and thus in forages, which represent a major component of ruminant diets. These phenolic acids are inhibitory to ruminal microorganisms (Chesson *et al.*, 1982; Borneman *et al.*, 1986; Akin *et al.*, 1993). Ruminal bacteria that utilize hemicellulose and cellulose may also produce aryl esterase (Mc Dermid *et al.*, 1990). This observation and the close association of cellulolytic bacteria with the particulate

fraction of the rumen suggest that these microorganisms are exposed to high local concentration of phenolic acid. Consequently, detoxyfication by degradation or modification of these aromatic compounds is under investigation.

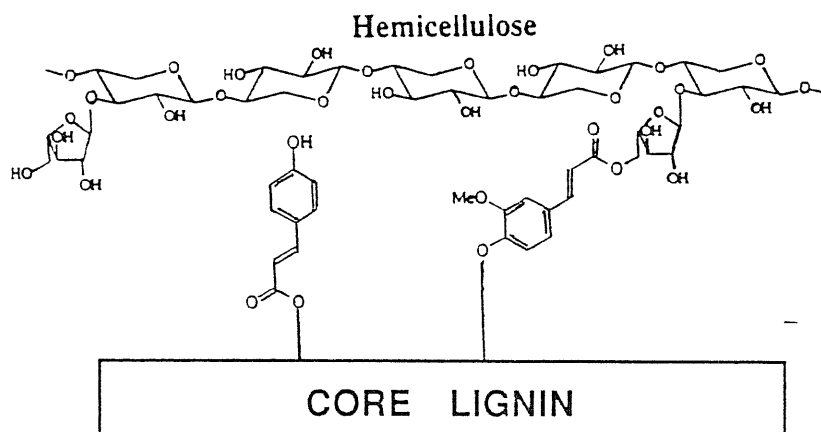


FIG.2 Schematic structure of cross-linkage of hemicellulose to core lignin via an ester-ether ferulic acid linked moiety. *p*-coumaric acid is shown as an ester of core lignin (Jung and Ralph, 1990)

1.5 Rumen microbial population

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 mycoplasmas 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 bacteria, or they have been isolated only on relatively few occasions under specialized conditions. Aerobic organisms isolated from ruminants are *Acinetobacter* sp., *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 et al., 1983). Rumen bacteria species isolated frequently are the anaerobes *Bacteroides* sp., *Streptococcus bovis*, *Ruminococcus*, *Butyrivibrio fibrisolvens*, *Wolinella succinogenes*, *Veilonella parvula*, *Eubacterium* sp., *Clostridium* sp. 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.

1.6 Genetics of rumen bacteria

Studies of genetic systems and the regulation of gene expression in the prevalently anaerobic rumen microorganisms are in their beginning. The transfer of fundamental genetic knowledge gained with anaerobic bacteria from other ecosystems to their rumen counterparts is desiderable, but it requires more taxonomics studies to establish the identity of some rumen species.

Shuttle vectors and naturally occurring, self transmissible plasmids have been developed for *B. ruminicola*. Streptococcal transposons have been introduced into *Butyrivibrio fibrisolvens* and *Streptococcus bovis*. Phage systems were also reported to be undergoing development for *S. bovis*.

DNA transfer mechanisms are being developed for the above vectors.

The cloning of ruminal bacteria genes in vitro and their expression in suitable host organisms has been used to discover new enzymes and characterize their functional components. For example, cellulase genes from *Bacteroides succinogenes* have been cloned and expressed in *E. coli* (Crosby et al., 1984). Genomic libraries have been constructed for the cellulolytic rumen bacteria *Ruminococcus albus* SY3 and *Butyrivibrio* A46 (Hobson, 1988)

1.7 Objective of this work

This thesis focuses upon the identification and characterization of modifying and degrading enzymes from ruminal bacteria active on monomeric lignin aromatic compounds, especially those involved in ferulic and *p*-coumaric acid decarboxylation by *B. pumilus*.. The identification of new enzymatic conversions of lignin model compounds could clarify the pathways of aromatic ring opening and offer the possibility to employ the organisms or their enzymes in the detoxification processes. The decarboxylation activity of *B. pumilus* was investigated and the related enzyme was characterized in the framework of the main objective of this research, which is to find out the gene encoding this enzyme. For this purpose a *B. pumilus* chromosomal DNA library was

constructed and a short amino-terminal sequence of its protein was used to design oligonucleotides to screen the library. The conditions of hybridization of these oligonucleotides have been optimized to improve the specificity of the hybridization. The screening of the DNA library is next step towards the goal of the project.

2. MATERIALS AND METHODS

2.1 Organisms and plasmids

2.1.1 Strains

B. pumilus PS213 isolated as described by Degrassi *et al.*, 1993

E. coli strains used were DH5α (*endA1, hsdR17, supE44, thi-1, recA1, gyrA96, relA1, ΔlacU169 (φ80lacZΔM15*)), (Hanahan, 1983) and XL-I *(endA1, hsdR17* (r_K^-, m_K^+), *supE44, thi-1, λ⁻, recA1, gyrA96, relA1, Δ(lac), [F', proAB, laclq ZΔM15, Tn10(*tet^R*)])* (Bullock *et al.*, 1987)

2.1.2 Plasmids

The following plasmids were used:

pBR322 (Bolivar *et al.,* 1977)

pRS426, a 5726 bp yeast shuttle vector which shares with pBluescript II SK+ the *LacZ* α–fragment for blue/white color screening, T7/T3 promoters for in vitro RNA transcription, and an f1 phage origin for production of single stranded DNA. It contains *URA3* marker and 2µ origin. (Christianson *et al.*, 1992).

2.2 Media

LB

1%	Bacto-Tryptone
1%	NaCl
0.5%	Bacto-Yeast Extract
pH 7.0	

Medium was sterilized by autoclaving for 20 minutes at 1.0 bar.

Stock solution of ampicillin was prepared at 50 mg/ml in redistilled water, sterilized by filtration through a 0.22 µm filter, and stored at -20°C.

Stock solution of tetracyclin was prepared at 5 mg/ml in 50% ethanol and stored at -20°C.

Ampicillin or tetracyclin were added to warm medium (50°C) after autoclaving at final concentration of 100 µg or 12.5 µg respectively for selection of recombinant plasmids.

20 mg/ml stock solution of X-gal was prepared in N-N- dimethylformamide and 100 µl were spread on each plate. 1M IPTG was prepared in water, filtered and 50 ul 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 was adjusted to 7.4 and medium autoclaved 20 minutes at 1 bar. 2 ml of filtered MgSO4 1M and 0.1 ml of filtered CaCl2 1M were added

Media for strictly or facultative anaerobic ruminal bacteria (ANA Medium)

(Modification of Calwell and Bryant, 1966)

0.05%	Glucose
0.05%	Pectin
0.05%	Cellobiose
0.05%	Soluble Starch

1%	Sodium lactate
0.05 %	Cysteine HCl H_2O
0.0001%	Resazurin
0.05%	Peptone
0.05%	Yeast Extract
0.0001%	Hemin
3.1ml/l	Volatile Fatty Acids**
500 ml/l	Mineral salt solution*
2.0%	Agar

pH 6.7 to 6.8

***Mineral salt solution**

0.2 g $CaCl_2$ (anhydrous)

0.2 g $MgSO_4$ (anhydrous)

1.0 g K_2HPO_4

1.0 g KH_2PO_4

10.0 g $NaHCO_3$

2.0 g NaCl

**** Volatile Fatty Acid Mixture:**

17 ml Acetic	(2.9×10^{-2} M)
6 ml Propionic	(8.0×10^{-3} M)
4 ml Butyric	(4.3×10^{-3} M)
1 ml Isobutyric	(1.1×10^{-3} M)
1 ml n-Valeric	(9.0×10^{-4} M)
1 ml Isovaleric	(9.0×10^{-4} M)
1 ml DL-a-Methylbutyric	(9.0×10^{-4} M)

The figures in parentheses indicate the final concentration of each acid when 3.1 ml of mixture is placed in a final volume of 1000 ml.

The medium was bubbled with O_2-free CO_2 gas for 15 min or more until the resazurin color in the medium turned pink and autoclaved 20 minutes at 1 bar. Before using, plates of solidified media were left overnight to stabilize in anaerobic atmosphere.

Yeast Extract Medium (YEM)

0.045%	K_2HPO_4
0.045%	KH_2PO_4
0.09%	NaCl
0.09%	$(NH_4)_2SO_4$
0.009%	$MgSO_4 \cdot 7H_2O$
0.009%	$CaCl_2$
0.1%	Yeast Extract
0.45%	Na_2CO_3
2.0%	Agar
0.0001%	Resazurin Sodium
0.025%	L-Cysteine Hydrochloride
0.025%	$Na_2S \cdot 9H_2O$

0.015-0.040% Glucose

pH 6.8

A mixed solution of minerals and Yeast Extract were stirred together with glucose and resazurin, an oxidation-reduction indicator, added Na_2CO_3, adjusted to pH 7.0 with NaOH, and kept at around 50 °C for 15 min. The medium was bubbled with O_2-free CO_2 gas for 15 min or more until the resazurin color in the medium turned pink. L-Cysteine was added, with the pH dropping to 6.7 to 6.8. The

volume was made up to 1000 ml, and Na_2S added finally to enhance the reductive state. Medium was autoclaved 20 minutes at 1 bar.

Fluid Thioglycollate medium (Difco)

1.5% Casitone

0.5% Yeast extract

0.55% Dextrose

0.25% Sodium chloride

0.05% L-Cystine

0.05% Sodium thioglycollate

0.075% Agar

0.0001% Resazurin

pH 7.0

Medium was sterilized in autoclave for 20 minutes at 121°C and dispensed into test tubes in anaerobic atmosphere

2.3 Lignin - related aromatic compounds

The lignin related aromatic compounds were purchased from either Sigma or Aldrich 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 (sinapinic acid), benzoic acid, *p*-hydroxybenzoic acid, protocatechuic acid, *p*-coumaric acid, *trans* -cinnamic acid, vanillin, vanillic acid. Vinylguaiacol was purchased from Lancaster.

All lignin related compounds were prepared as 5 gr/l stock solutions, filter sterilized and added to the autoclaved media.

2.4 Culture methods

2.4.1 Ruminal bacteria isolation and identification

Bacterial strains were isolated on ANA Medium and YEM from ruminal fluid collected as described in Degrassi *et al.*, 1993. To test if the strains were strictly anaerobic, aerotolerant or facultative, each strain was streaked, after growth in anaerobiosis, on an ANA and LB plate and incubated in aerobiosis at the same temperature for 24-48 hrs.

A Gram stain was made for each strain. The cell morphology and the colony morphology were described, as well as Gram stain result and the modality of the growth.

An identification test was made using API kits (bioMérieux, Italia) according to the information obtained by the previous analysis. Anaerobic and aerotolerant strains were tested with RAPID ID 32 A kit, while the anaerobic facultative strains were tested with API 50 CH kit using the required inoculum medium API 50 CHB/CHE.

2.4.2 Strain conservation

Each strain was stored at -80°C with 15% glycerol after 48 hours of growth in fluid thioglycollate medium.

2.5 Analytical methods

2.5.1 Analysis of degradation pattern of isolated ruminal bacteria

Each strain was inoculated in 3 ml of Fluid Thioglycollate medium. After 48 hours of incubation at 37°C in anaerobic chamber, 100 µl of culture were transferred into 5 ml of the same medium containing 0.5 mg/ml of lignin model compound. After 48 hours of incubation under agitation at 37°C in anaerobiosis 50 µl of

culture were collected and diluted 20 times in 0.2M Tris buffer pH7. After centrifugation to remove cells the degradation was tested by comparing the UV spectra of each lignin model compound before and after growth. The same procedure was followed to test degrading activity in aerobiosis, but LB medium was used instead of thioglycollate.

2.5.2 Test of inducibility of the ferulic-decarboxylase gene in *Bacillus pumilus*

20 ml of LB and 20 ml of LB plus 0.5 gr/l ferulic acid were inoculated respectively with 100 µl of a starter culture of *B. pumilus* and incubated overnight at 37°C. The following day each culture was centrifuged and the pellet resuspended in 20 ml of fresh LB medium plus 0.5 gr/l ferulic acid to the same optical density. The utilization of ferulic acid was checked at different intervals at the spectrophotometer comparing the spectra obtained between 200 and 400 nm from the induced and non induced culture.

2.5.3 Preparation of cell extracts

Bacillus pumilus was grown in batches of 150 ml contained in 1-litre flask at 37°C. The growth medium was LB and LB containing 0.5 gr/l of ferulic acid. Cells were collected by centrifugation at 8,000 rpm in a Sorvall GSA rotor and washed in 50 mM Tris-HCl pH 7.6. The pellet 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 (Penalava and Salas, 1982). After incubation on ice for 1 hour the sample were frozen in dry ice, then thawed at 37°C for half an hour or till the suspension became clear.

2.5.4 Protein determination

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

2.5.5 Enzyme activity assay

The assay mixture was constituted by 50 mM sodium phosphate buffer pH 6 and 0.5 gr/l of ferulic acid. For testing the activity of the enzyme in anaerobiosis the mixture was degassed and stabilized some hours in anaerobic atmosphere. Reactions were started by adding cell extract (10%) and incubating at 37°C in aerobiosis and anaerobiosis respectively. In the latter case the cell extract was degassed in speed vacuum for few seconds. 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 observing the appearance of a new peak at 258 nm with the spectrophotometer and checking with HPLC at λ=260, with a flow of 0.5 ml/min and MeOH/H2O (H+) 60/40 as mobile phase, the appearance of a peak having the retention time of 4-vinyl-guaiacol and disappearance of the peak corresponding to ferulic acid.

2.6 DNA techniques

2.6.1 Large scale chromosomal DNA purification

Total DNA extraction and chromosomal DNA purification was carried out as a modification of the protocol described by Ausubel *et al.*, 1987. 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 (Du Pont, Wilmington, Delaware) centrifuge in 250 ml bottles using a GSA rotor at 4000xg 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 6000xg in a Sorvall RC-5B (Du Pont, Wilmington, Delaware) centrifuge using a SS-34 rotor at 4000xg at room temperature. The aqueous supernatant was transferred to a 50 ml Falcon tube with a wide-bored pipet. 0.6 volumes of isopropanol were added and it was mixed until a stringly white DNA pellet precipitated out of solution and condensed into a tight mass. The precipitate was transferred to 1 ml of 70% ethanol in a fresh 2 ml Eppendorf tube, by hooking it on the end of a Pasteur pipet that was bent and sealed in a Bunsen flame. Pellet was spun 5 minutes at 14000 rpm in an Eppendorf Centrifuge 5415 C. 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 Falcon 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 RT600B centrifuge and avoiding the floating debris was dispensed into a 12.5 ml polyallomer Quick-seal Beckman centrifuge tube. It was spun 8 hours at 54K rpm in Beckman L8-70M Centrifuge using the vertical rotor Vti65.

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 n-butanol (saturated with water). One volume of n-butanol was added to the recovered DNA solution, thoroughly mixed, centrifuged at 2000 rpm in a Sorvall RT600B centrifuge for 3 minutes at room temperature. The lower

aqueous phase was recovered and 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 1.8 as a pure DNA preparation (Sambrook *et al.*, 1989). DNA was stored at 4°C until needed.

2.6.2 Large scale plasmid DNA purification

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, Wilmington, Delaware) centrifuge in 250 ml bottles using a GSA rotor at 4000xg at 4°C for 10 minutes. Pellet was resuspended completely in 10 ml TE (50 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0) containing 100 µg/ml RNAse. 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, 1983). 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 and shorter plasmid DNA reannealing 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 QUIAGEN-tip 500 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 Quiagen Kit. DNA was precipitated with 0.7 volumes of isopropanol, previously equilibrated at room temperature and centrifuged in a Sorvall RC-5B (Du Pont, Wilmington,

Delaware) centrifuge at 15000xg, at 4°C for 30 minutes. Surnatant was carefully removed. The pellet was dried and resuspended in TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0).

2.6.3 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. 2 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 surnatant was almost completely removed, leaving approximately 100 μl of surnatant 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. Supernatant was transferred to 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. Supernatant was discarded, pellet was washed with 70% ethanol and dried under vacuum. The pellet was resuspended in 40 ml of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) and stored at 4°C until needed. RNA was degraded during incubation with restriction endonucleases by the addition to the reaction mixture of 25 μg/ml RNase.

2.6.4 Total and partial 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 0.3 volumes of agarose gel running buffer and then 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.

Partial digestion of high-molecular-weight DNA was obtained by incubation with limiting amounts of a particular restriction enzyme for variable lenghts of time. Samples from the digested DNA were removed at different time intervals and analyzed by agarose gel electrophoresis to determine the average lenght of the digested DNA. The time points of digestion that were most enriched for the desired size fractions were then used as the guide for the preparative digestion of the same DNA. Partial digestion was generally tested using 10 μg of DNA in 100 μl of reaction volume with a quantity of enzyme necessary to digest 20% of the total DNA. 5 μl of reaction mixture were collected every 5-10 minutes and reaction blocked adding EDTA to a final concentration of 10 mM. These aliquots were tested in a 0.5% agarose gel. After defining the right time point, digestion was scaled up 10 times.

2.6.5 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 mold and let solidify. Once the gel solidified it was placed into the gel apparatus and submerged with 0.5xTBE buffer. DNA samples were loaded on the wells after having added 1/6 volume of 6x DNA sample buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Fycoll in water). 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.

2.6.6 Sucrose gradient fractionation of digested DNA

To separate a mixture of DNA fragments ranging in size from hundreds of base pairs to over 100,000 bp obtained with partial digestion, a discontinuous 10% to 40% (w/v) gradient was prepared as suggested by Sambrook, *et al.*, 1989. The 12 ml discontinuous gradient was constructed in a polyallomer Beckman centrifuge tubes loading first the heaviest concentration, followed by the lighter concentrations. Once the gradient was ready the sample (less than 500 μl) was carefully layered on the top. No more than 0.2 mg of digested genomic DNA were loaded to allow a good separation of different size DNA fragments. The sucrose gradient was centrifuged at 20°C, 26,000 rpm, 26 hours, in Beckman L8-70M Centrifuge using the swing rotor SW-41.

0.5 ml fractions were collected through a 18-gauge needle inserted through the bottom of the centrifuge tube. 25 μl of each fraction were analyzed by electrophoresis through a thick 0.5% agarose gel using as a marker 1 Kb DNA ladder and adjusting its salt and sucrose concentrations to match those of the samples. After photographing the gel, locate and pool the fractions containing DNA fragments of desired size, DNA was recovered by precipitation with ethanol after having diluted the sample so that the concentration of sucrose is reduced to about 10%.

2.6.7 DNA ligation reactions

Ligation of DNA fragments to plasmid vector was essentially performed as described by Sambrook *et al.*, 1989. Ligation reactions were incubated at 4°C overnight and were typically performed in as small volume as possible 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. 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* for transformation, or an aliquot first analyzed by agarose gel electrophoresis to visualize the formation of higher molecular weight ligation products.

2.6.8 Dephoshorylation of linearized plasmid DNA

1-20 μg of linearized plasmid DNA were incubated in 50 μl of reaction mixture with 0.1U of alkaline phosphatase and incubated 30 minutes at 37°C. Enzyme was inactivated at 85°C for 15 minutes.

2.6.9 Preparation of competent 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 screened by replica plating on medium containing the second selective marker .

2.6.10 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 redissolved in 500 µl of sodium phosphate buffer pH 6.8. 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 in a 1 cm path-lenght cuvette.

2.6.11 Oligonucleotides 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 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). 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^{TM} column.

2.6.12 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 (3.6M NaCl, 0.2M sodium phosphate, 0.02M EDTA pH 8) and a nylon membrane Hybond^{TM} - N+ (Amersham) of the size of the gel was placed over the gel escluding 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 over night.

2.6.13 Filter hybridization

Filter was incubated at 25°C for 1-2 hours with 0.2 ml/cm^2 of hybridization solution (5X SSPE, 5X Denhardt's solution, 10% w/v SDS). After pre-hybridization 1ng/ml of labelled oligonucleotide was added and incubated overnight at room temperature. Filter was washed extensively at room temperature with 6XSSC (1 M NaCl, 0.1 M Na$_3$citrate) and then briefly 5-10 minutes at 30°C.

3. RESULTS

3.1 Characterization of bacterial strains isolated from ruminal fluid

Thirty bacterial strains were isolated from ruminal fluid in anaerobic atmosphere and distinguished on the basis of the different morphologies of their colonies and their different cellular shape. These strains were investigated further with the appropriate API kits after having tested if they were strictly or facultative anaerobic. For this purpose they were streaked on ANA plates in anaerobiosis and on the same medium prepared without Cys, which is usually used as a reductive agent, and CO_2 bubbling, in aerobiosis. Growth in aerobiosis was also tested on LB medium.

A comparison of the patterns obtained using the enzymatic tests of the API ID32A kit designed for strictly anaerobes and the study of the carbohydrate metabolism of the facultative anaerobes obtained with API 50CH kit allowed to reduce the number of the different strains to 18.

The 18 strains were analyzed microscopically for Gram reaction and cell morphology. All these microorganisms were gram-positive, suggesting an involuntary selection introduced in the first step of isolation.

Seven strains were facultative anaerobes and morphologically similar to *Bacillus* sp. or cocci in groups. The anaerobic or aerotolerant microorganisms were prevalently cocci, but it was often difficult to define correctly the shape of the cells since they were extremely small.

3.2 Degradation activity of the isolated strains

The eighteen bacterial stains, biochemically and morphologically characterized, were tested for their ability to utilize fourteen monomeric lignin model compounds: ferulic acid, syringic acid, syringaldehyde, 3,4-dimethoxy-benzaldehyde (veratryl aldehyde), 3,4-dimethoxybenzyl acid (veratric acid), 3,5-dimethoxy-4-hydroxycinnamic (sinapinic acid), benzoic acid, *p*-hydroxybenzoic

TABLE 1 - DEGRADATION PATTERN

STRAINS	SYR.AC.	SYR.ALD.	VERATRYL.ALD.	p-HYDR.BENZ.AC.	VERATRIC AC.	SINAPINIC AC.	BENZOIC AC.	PROTOCATECHUIC AC.	VINYLGUAIACOL	FERULIC AC.	p-COUMARIC AC.	tCINNAMIC AC.	VANILLIN	VANILLIC AC.
1	-	-	-	+	-	+	-	-	+	+	+	-	-	+
2	-	-	-	+	-	+	-	-	+	+	+	-	-	+
3	-	-	-	-	-	+	-	-	+	-	-	-	-	-
4	-	-	-	-	-	+	-	-	+	-	-	-	-	-
5	-	-	-	-	-	+	-	-	+	-	-	-	-	-
6	-	-	-	-	-	-	-	-	+	+	+	-	-	-
7	-	-	-	-	-	-	-	-	+	+	+	-	-	-
8	-	-	-	-	-	-	-	-	+	+	+	-	-	-
9	-	-	-	-	-	-	-	-	+	+	+	-	-	-
10	-	-	-	-	-	-	-	-	+	-	-	-	-	-
11	-	-	-	-	-	+	-	-	+	-	-	-	-	-
12	-	-	-	-	-	+	-	-	+	-	-	-	-	-
13	-	-	-	-	-	+	-	-	+	-	-	-	-	-
14	-	-	-	-	-	+	-	-	+	-	-	-	-	-
15	-	-	-	-	-	+	-	-	+	-	-	-	-	-
16	-	-	-	-	-	+	-	-	+	+	+	+	-	-
17	-	-	-	+	-	+	-	-	+	+	+	-	-	+
18	-	-	-	-	-	-	-	-	+	+	+	-	-	-

(+) modified U.V. spectrum; (-) unmodified U.V. spectrum.

acid, protocatechuic acid, *p*-coumaric acid, *trans* -cinnamic acid, vanillin, vanillic acid and vinylguaiacol

Each strain was grown in anaerobiosis, 48 hours, in 5 ml of Thioglycollate medium containing 0.5 mg/ml of the interesting compound. According to UV spectrophotometric data no one of the 14 aromatic compounds were completely mineralized anaerobically in the time of investigation.

Syringic acid, syringaldhehyde, veratryl aldehyde, veratric acid, benzoic acid, protocatechuic acid and vanillin did not show any significant modification of their UV spectra.

Twelve of the eighteen tested strains showed ability to degrade sinapinic acid. Degradation of ferulic acid by nine strains was strictly associated with the degradation of *p*-coumaric acid.

While the UV spectrum of vanillin remained unmodified after 48 hours of incubation with all the strains analyzed, vanillic acid was degraded by three facultative anaerobic microorganisms, the same which showed activity toward p-hydroxybenzoic acid. *Trans*-cinnamic acid was actively degraded by an aerotolerant strain. All four strains were also able to degrade sinapinic, ferulic and *p*-coumaric acid.

The concentration of vinylguaiacol was reduced in the medium after incubation and this phenomenon correlated with no appearance of new absorption peaks, could be explained by the uptake of this aromatic compound by the cells.

(Table 1).

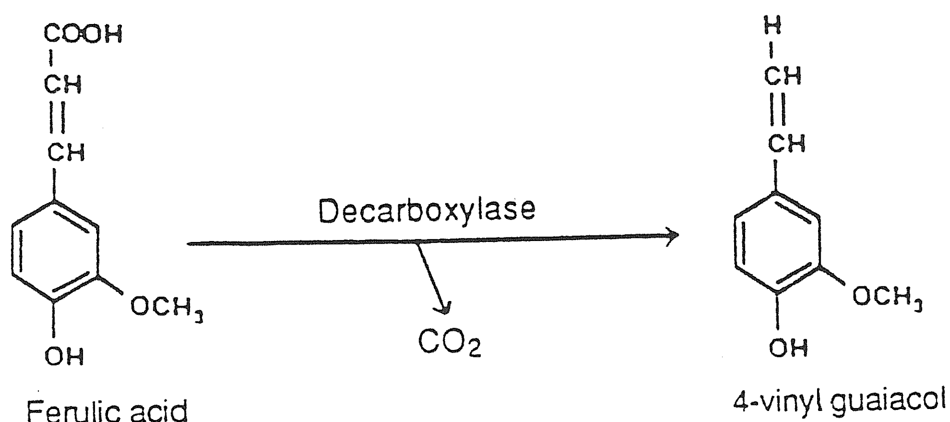
3.3 Degrading activity of a strictly aerobic ruminal *Bacillus* strain

B. pumilus PS213, a strictly aerobic microorganism previously isolated from cow rumen fluid and screened for the degradation of lignin related aromatic compounds, was demonstrated to be able to degrade ferulic acid, *p*-coumaric acid and veratryl aldehyde. *p*-coumaric acid and ferulic acid were the best substrates for the organism to degrade within 1 and 2 days in aerobiosis with the

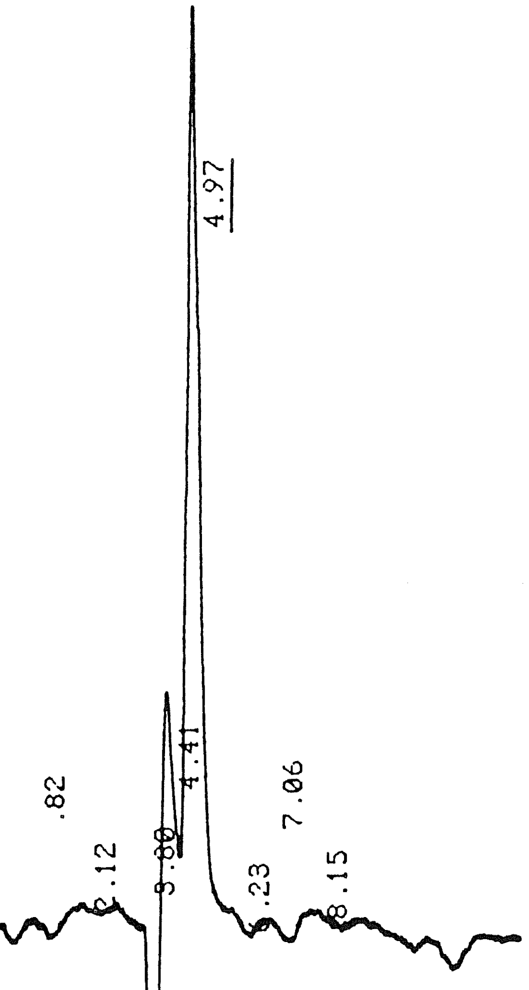
evident appearance of a new compound. Ferulic acid and *p*-coumaric acid were degraded and products of degradation were accumulated as shown by a change in the UV spectrum of the maximum wavelenght of 285/310 to 260 nm with a shoulder between 285 and 310 nm for ferulic acid, and 285 to 258 nm with a shoulder between 285 and 310 for *p*-coumaric acid. These UV spectra modifications overlapped those observed for the anaerobic microorganisms showing activity toward these two aromatic model compounds.

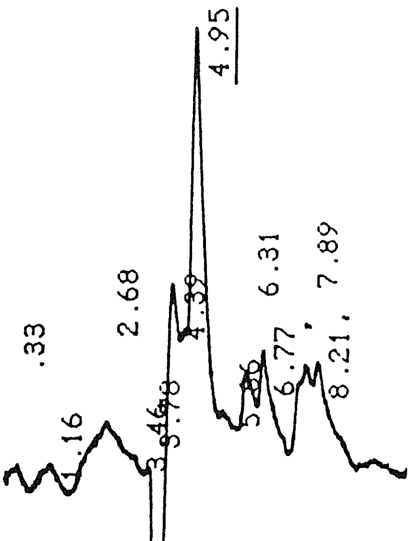
3.4 Decarboxylase activity in *B. pumilus* PS213

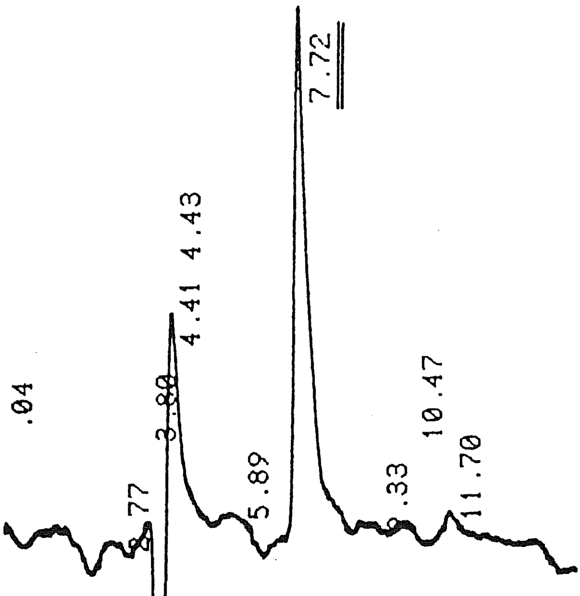
Following the shift in UV absorption of the maximum wavelenght of ferulic acid it was suggested that the resulting product could be 4-vinylguaiacol. In order to confirm the transformation of ferulate to 4-vinylguaiacol by *Bacillus pumilus* , the elution time of 4-vinylguaiacol extracted from a *Fusarium solani* culture supernatant (Nazareth and Mavinkurve, 1986) was compared to that of the unknown peak of *B. pumilus* M9CA medium supernatant by HPLC analysis. The elution time was exactly the same confirming the identity of the spectra and the characteristic smell of the product (Fig. 3). This was the evidence for the presence of a ferulate decarboxylase activity in *B. pumilus* .



The ferulate decarboxylase activity in crude, cell-free extracts prepared from *B. pumilus* grown in LB medium plus 0.5 gr/l ferulic acid was demonstrated







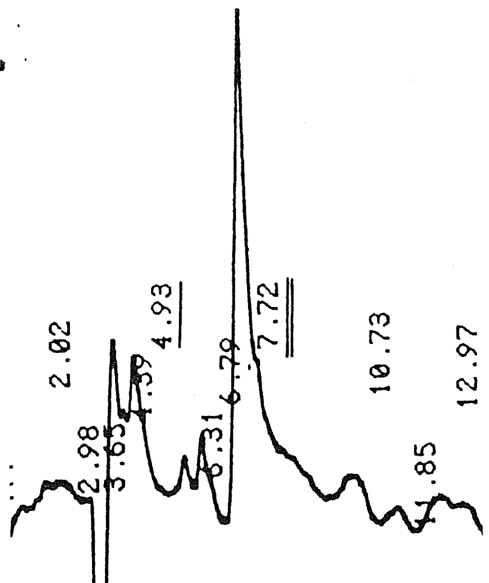


FIG.3 HPLC retention time of ferulic acid (A, B) and 4-vinyl guaiacol (C, D) at λ=260 nm and flow rate=0.5 ml min^{-1} (A) ferulic acid 0.5 µg ml^{-1}; (B) M9CA medium plus 0.5 µg ml^{-1} ferulic acid; (C) 4-vinyl guaiacol extracted from *F. solani* culture supernatant 0.5 µg ml^{-1}; (D) M9CA medium plus 0.5 µg ml^{-1} after growth of *B. pumilus* overnight.

following the shift in UV absorption of the maximum wavelenght of the compound in the assay mixture and observing that the spectrum changed as in the whole cell containing culture. The results were confirmed by HPLC showing the appearance of a new peak having the retention time of 4-vinyl guaiacol (Fig. 4). To check if the enzyme was able to work also in anaerobiosis the tests were repeated adding the same amount of protein to the reaction mixture stabilized four hours in anaerobic chamber. Moreover the cell extract was degassed to eliminate O_2 eventually present. The decarboxylase enzyme showed to be able to work also in the absence of O_2 (Fig. 5). This result was further confirmed adding 0.025% Cys and 0.001% resaruzin to the reaction mixture. The mixture was incubated in anaerobic atmosphere until the resaruzin indicator changed from pink to incolor, to ensure the condition of anaerobiosis before and after having added the cell extract. The cell free extract prepared from a strain grown in the absence of ferulic acid was used as a control.

A test of inducibility for the decarboxylase activity was performed as described in material and methods. The cells grown in the presence or absence of ferulic acid were centrifuged and resuspended in LB plus the aromatic compound to the same $O.D._{600}$ of 2.3. After only 10 minutes a big difference in the rate of degradation of ferulic acid by the induced culture in comparison to the non induced one was observed. This difference was less and less relevant in the following three controls made every 20 minutes, while the optical density was increasing really 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 was 3 and the two spectra overlapped (Fig. 6).
The inducibility of the decarboxylase gene was thus demonstrated, though the low activity in aerobiosis and anaerobiosis of the cell free extract, obtained from a culture in the absence of ferulic acid and used as a negative control in the

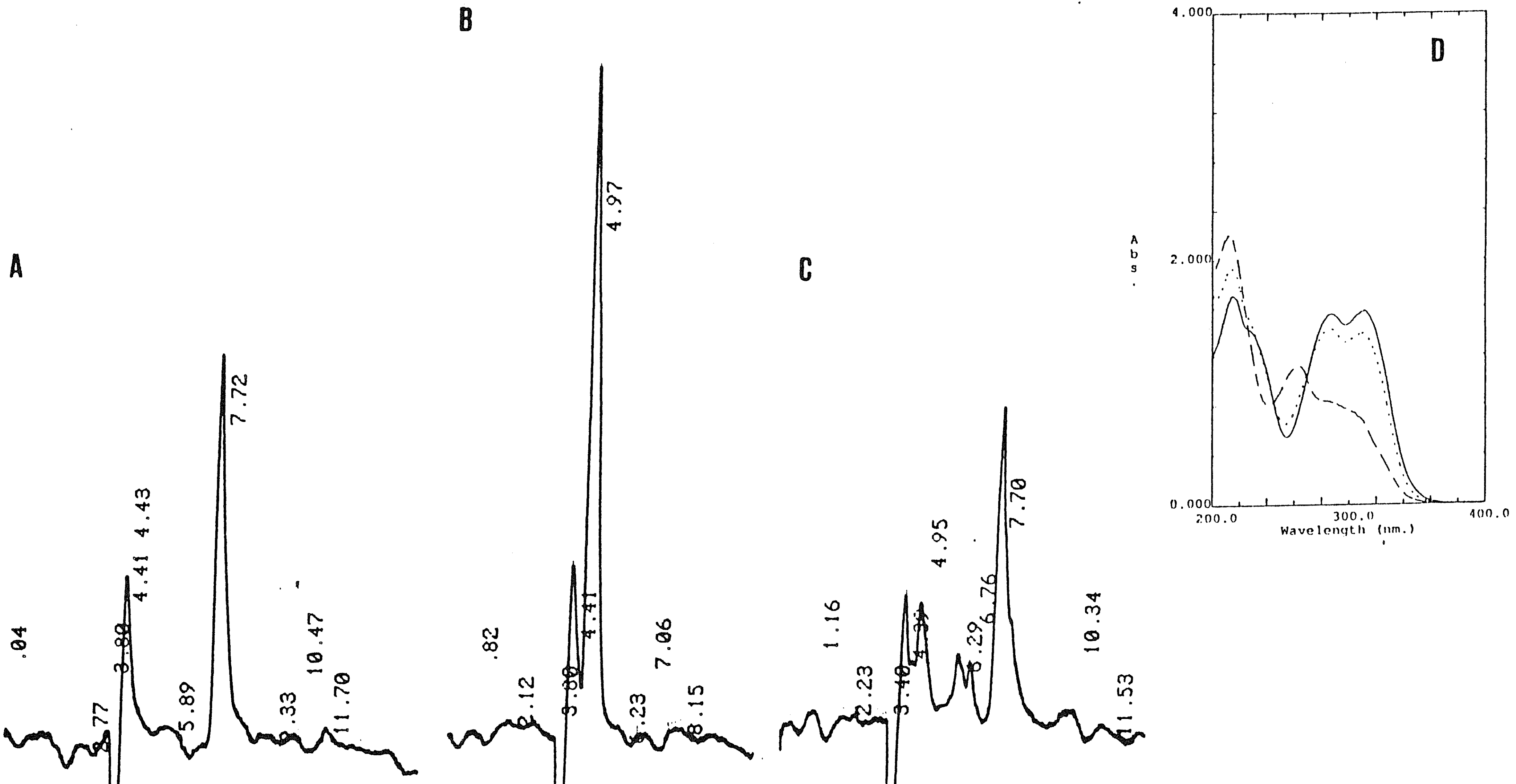


FIG.4 HPLC retention time of 0.5 µg ml^{-1} 4-vinyl guaiacol (A) and 0.5 µg ml^{-1} ferulic acid (B) at λ=260 nm and flow rate=0.5 ml min^{-1}. (C) 50 mM phosphate buffer pH 6 plus ferulic acid after incubation with *B. pumilus* cell extract in aerobiosis.(D) UV spectra of ferulic acid after incubation in aerobiosis with cell extract of *B. pumilus* grown in LB plus ferulic acid (----) or in LB

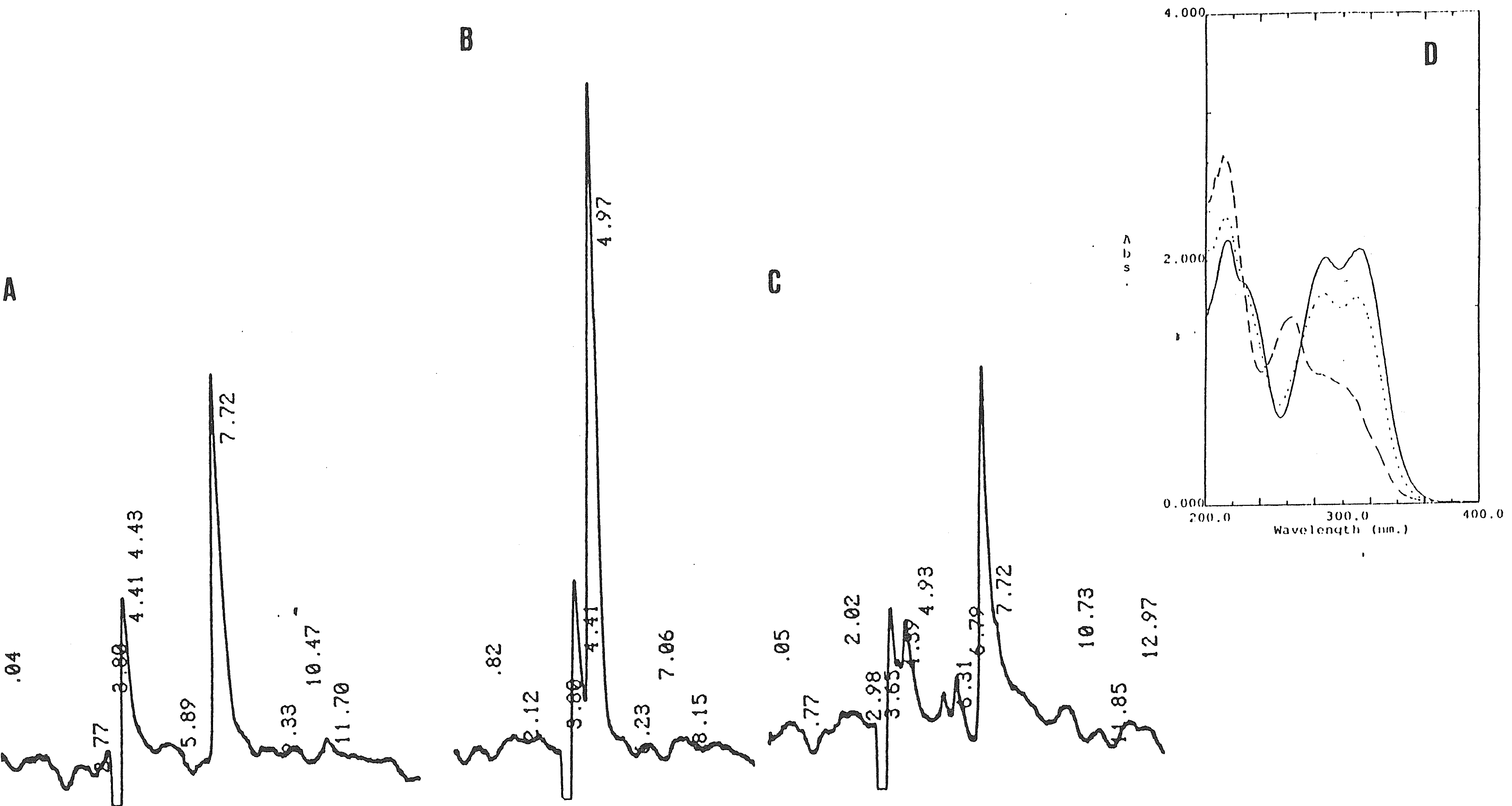


FIG.5 HPLC retention time of 0.5 µg ml^{-1} 4-vinyl guaiacol (A) and 0.5 µg ml^{-1} ferulic acid (B) at λ=260 nm and flow rate=0.5 ml min^{-1}. (C) 50 mM phosphate buffer pH 6 plus ferulic acid after incubation with *B. pumilus* cell extract in anaerobiosis. (D) UV spectra of ferulic acid after incubation in anaerobiosis with cell extract of *B. pumilus* grown in LB plus ferulic acid (----) or in LB without ferulic acid (.....) ; control (—).

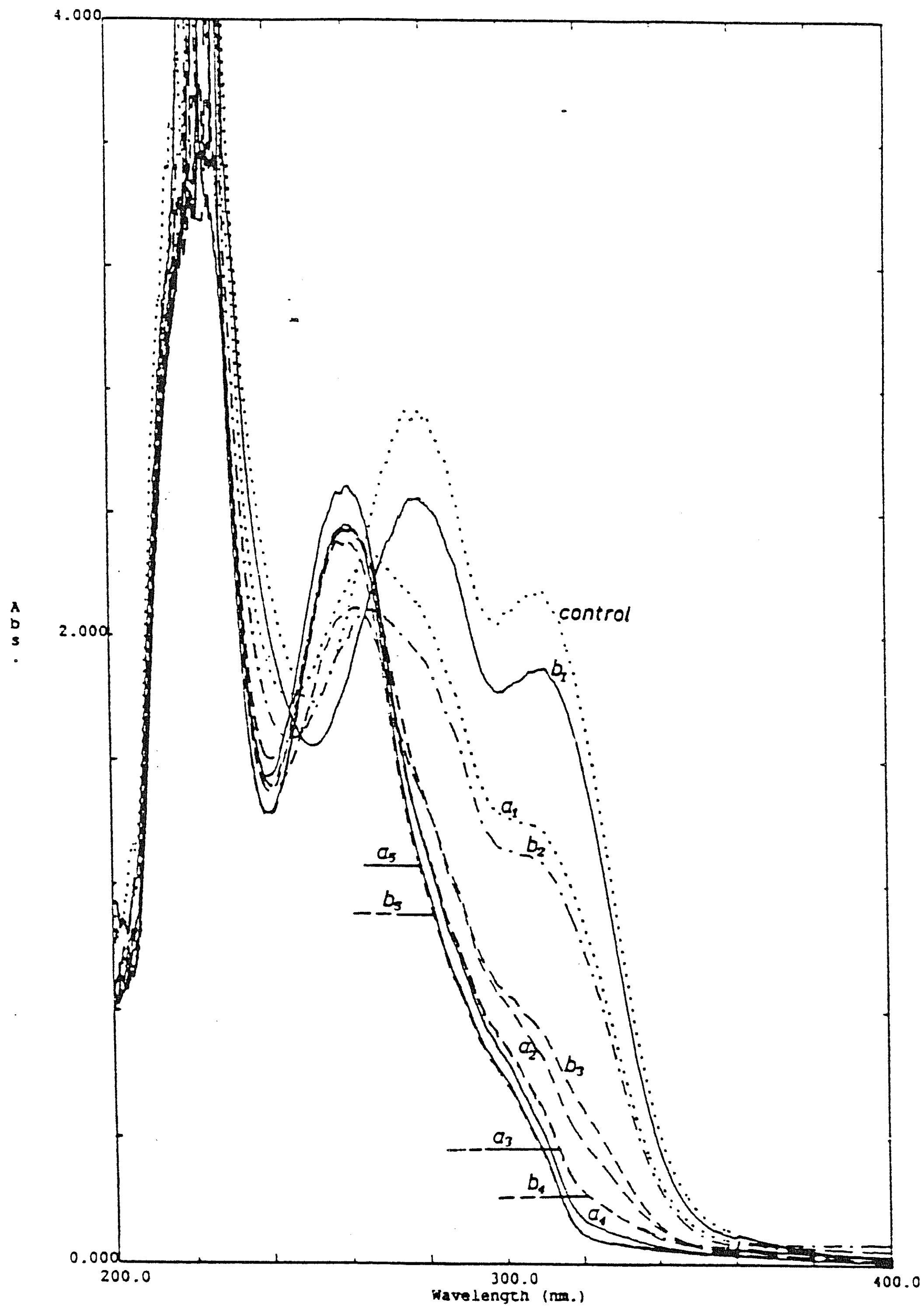


FIG.6 Test of inducibility of the decarboxylase activity.

Control = LB plus 0.5 mg ml^{-1} of ferulic acid. a = degradation 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).

previous experiment, showed that there is always a low constitutive expression of enzymatic activity, which is induced by the substrate.

3.5 *B. pumilus* PS213 DNA library

Chromosomal DNA was extracted and purified as described in materials and methods. The first approach was to partially digest DNA with Sau3AI restriction enzyme to clone it in a BamHI site. Partial digestion was optimized and the ideal time of digestion was discovered to be 30 minutes using 0.8 U of Sau3AI and about 100 μg of DNA in 1 ml of reaction mixture. These conditions were used for the preparative partial digestion followed by sucrose gradient fractionation. After collection of the fractions and analysis on agarose gel, the fraction number 9 (Fig. 7), containing 10 kb fragments, was chosen.

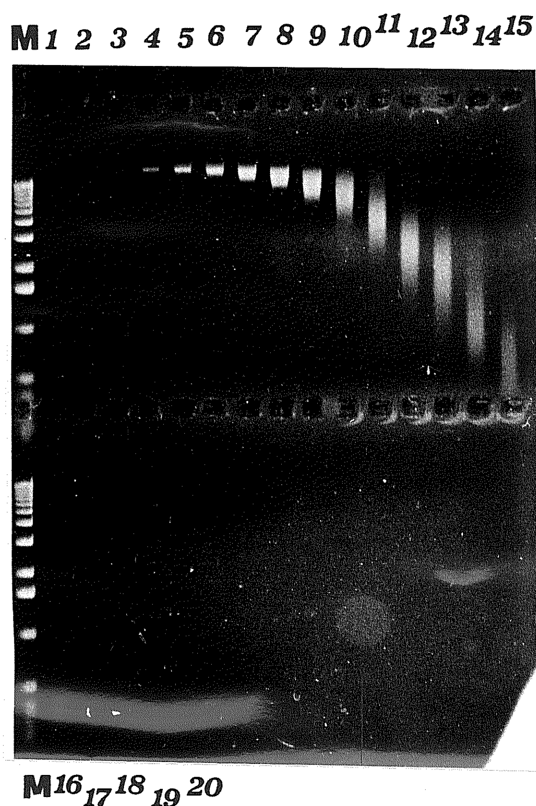


FIG.7 Sucrose-gradient of *B. pumilus* DNA digested with Sau3AI. M = 1 Kb DNA ladder, 1-20 = fractions.

DNA recovered from this fraction was cloned in the pRS426 plasmid linearized with BamHI restriction enzyme and dephosphorylated with calf intestinal phosphatase. The ratio plasmid:DNA used in the ligation reaction was 1:3. The ligation mixture was used to transform *E. coli* XL-1 and a large number of white colonies were obtained on LB plus ampicillin, X-gal and IPTG. In the positive control, prepared using digested and religated plasmid without insert, only very few white colonies were detected. Mini preps were made, but after digestion with BamHI no inserts were observed.

A control experiment was performed using the same plasmid and cloning in the same site an excess of *B. pumilus* DNA totally digested with BamHI. The result was again negative and no one white colony was detected.

Since the classical approach did not work, *B. pumilus* DNA was digested with PstI restriction enzyme and cloned in pBR322 plasmid linearized with PstI and dephosphorylated. This construct was used to trasform *E. coli* DH5α, as described by Panbangred *et al.*, 1983. About one thousand colonies contained inserts and they were able to grow on LB plus ampicillin, but were not able to grow on LB plus tetracyclin according to the fact that insertions in PstI site inactivate TetR gene. However the same plasmid had already been used unsuccessfully in the same strain to clone in BamHI site *B. pumilus* DNA partially digested with Sau3AI restriction enzyme.

To increase the probability to have the whole gene in one fragment and a good probability of having represented the whole *B. pumilus* genome in the library, partial digestion of *B. pumilus* DNA with PstI was performed. Optimized conditions of partial digestion were 10 μg of DNA in 100 μl of reaction mixture incubated 25 minutes with 4 U of PstI. The reaction was scaled up 10 times for preparative partial digestion. Sucrose gradient was performed using always the

same conditions and the fraction number 13 (Fig. 8) was detected to contain DNA fragments with an average size of 10 Kb.

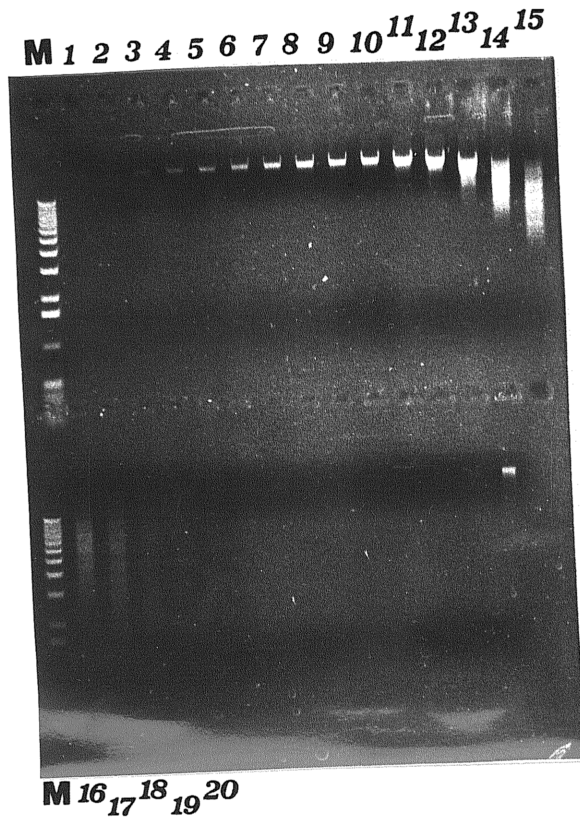


FIG.8 Sucrose gradient of *B. pumilus* DNA digested with PstI. M = 1 Kb DNA ladder, 1-20 = fractions.

After cloning of the selected DNA fragments in PstI site of pBR322, previously linearized and dephosphorylated, transformation in *E. coli* DH5α was performed. Mini preps showed successful cloning of partially digested DNA of the selected size (Fig. 9). About two thousand positive clones were collected, enough to give a good representation of the B. *pumilus* genome.

M c 1 1' 2 2' 3 3' 4 4' 5 5' 6 6' 7 7' 8 8' M

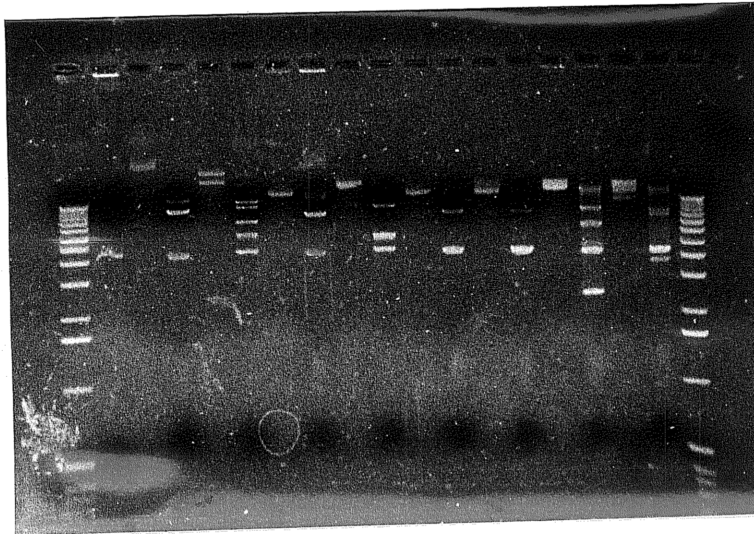


FIG.9 PstI digested plasmidic DNA from positive clones. M = 1 Kb DNA ladder, 1-8 = undigested DNA, 1'-8' = digested DNA, c = pBR322 linearized with PstI.

3.6 Optimization of oligonucleotide hybridization conditions

The decarboxylase enzyme from *B. pumilus* was purified in our laboratory by a three steps purification procedure and the determination of the N-terminal aminoacid sequence was performed by automated Edman degradation (Degrassi and Bruschi, 1993). A sequence of 9 aminoacids was determined, therefore the second and seventh aminoacids were difficult to identify and they were suggested to be serine and arginine respectively. The sequence was the following: M S(?) Q P V D R(?) H Y.

The first six aminoacids were used to design corrisponding oligonucleotides with the program BACKTRANSLATE (Wiscosin GCG) using the GCG CodonFrequencies of *Bacillus subtilis* (105 genes found in GenBank63). The second not very probable serine was translated with three inosines. Inosines were also used in the third position of the codons encoding the proline and the valine residues, where the probability among the four nucleotides was the same.

With this strategy, instead of using a pool of degenerated oligonucleotides, only four oligonucleotides were constructed and singularly tested:

OLIGO 1	5'A T G I I I C A A C C I G T I G A T3'
OLIGO 2	5'A T G I I I C A A C C I G T I G A C3'
OLIGO 3	5'A T G I I I C A G C C I G T I G A T3'
OLIGO 4	5'A T G I I I C A G C C I G T I G A C3'

After purification each oligonucleotide was terminally labelled and used as a probe. Southern blot analysis of *B. pumilus* genomic DNA digested to completion with PstI restriction enzyme showed that the oligonucleotides 3 and 4 hybridize with the same discrete band. These two oligonucleotides differ only for the 3' terminal nucleotide (G or C) and recognize a band of >12 Kb in digested *B. pumilus* DNA, but do not hybridize with PstI digested genomic DNA of *E. coli* DH5α, which is the host strain used for constructing the library (Fig. 10).

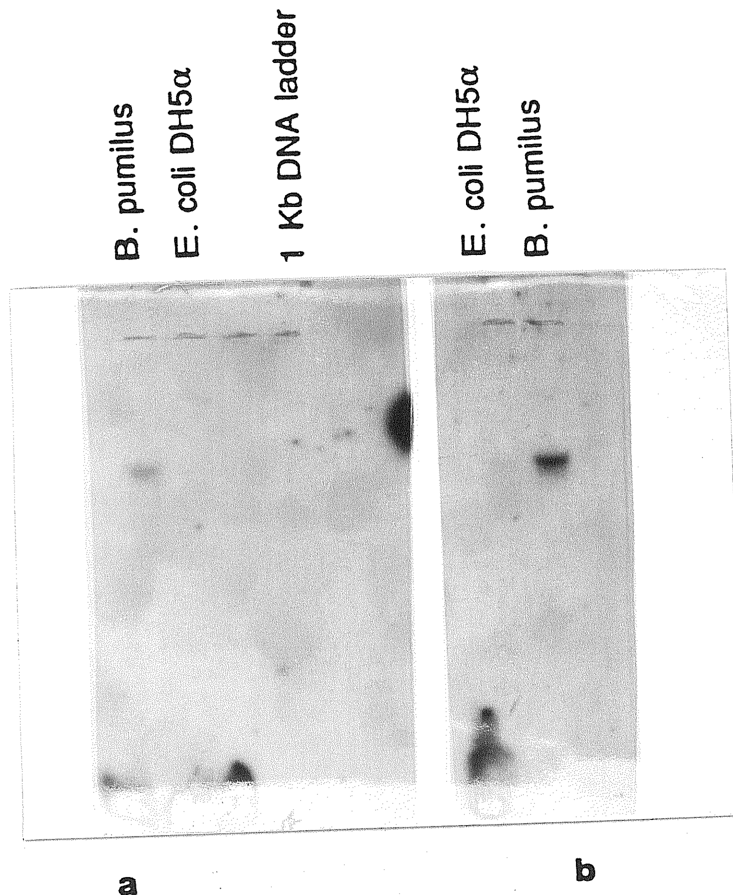


FIG.10 Southern hybridization with labelled (a) oligo 4 and (b) oligo 3.

4. DISCUSSION

Phenolic acids are common constituents of forage fed to ruminants. Particularly widespread are substituted cinnamic acids (p-coumaric, ferulic and sinapinic acid) closing related to the corresponding lignin monomers. Hydroxycinnamic acids often occur esterified to polysaccharides. It has been suggested that these compounds are released during ruminal anaerobic degradation of lignin or other phenolic constituents of the plant cell walls (Healy, 1980). The relatively easy cleavage of ester bonds in the non core lignin, compared to the C-C and ether linkages in core lignin, and the production of aryl esterases by rumen bacteria and fungi (McDermid *et al.,* 1990) suggest that these aromatic monomers are readily liberated in the rumen. Phenolic monomeric constituents of lignin have also been found to be inhibitors of growth of ruminal bacteria and their cellulose and hemicellulose degradation activity. Therefore, these compounds have a nutritional significance since they are considered an anti-quality factor in the forage and their removal is one of the reasons for the sodium hydroxide treatment of straws. We have shown that a microbial consortium from cow rumen fluid is able to convert several aromatic monomers (Degrassi et al., 1993) and perhaps this can have an important detoxicity role. Microorganisms present in the sheep rumen fluid decarboxylate 4-hydroxybenzoic acid and 4-hydroxy-phenylacetic acid and demethoxylate 3-methoxy-4-hydroxy-benzoic acid (vanillic acid), while a slight decarboxylation of 4-hydroxy cinnamic acids have been detected (Martin,1978).

In this study I isolated and characterized some microorganisms that in the previously analyzed rumen microbial consortia could be responsible for the anaerobic utilization of ferulic acid, *p*-coumaric acid, vanillic acid, *p*-hydroxybenzoic acid and sinapinic acid. Perhaps incubation was too short to detect the utilization of veratrylaldehyde, *trans*-cinnamic acid, vanillin, veratric acid, syringaldehyde and syringic acid observed in the longer incubation of

rumen consortia with these model compounds. This could also be an explanation for the lack of mineralization of these aromatic compounds, which often requires longer incubation times and perhaps is the product of microbial co-operation. Anyway further investigation is necessary, in particular degradation products analysis, 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 significantly 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. Strains isolated were prevalently aerotolerant and the facultative anaerobes showed activity towards several substrates. One strain, apparently aerotolerant, was particularly interesting because of its preferential modification of the cinnamic compounds and lack of activity towards benzoic compounds.

Modification of ferulic and *p*-coumaric acid has been observed to be widely spread among rumen strains. Ferulic acid is completely degraded in anaerobic conditions and in anaerobiosis by different microorganisms and possible pathways of ferulate metabolism have been proposed (Grbic-Galic, 1986; Grbic-Galic, 1985; Grbic-Galic and Young, 1985; Healy *et al.*, 1980; Nazareth and Mavinkurve, 1986). A *B. pumilus* strain, repeatedly isolated from rumen fluid, was demonstrated to be able to modify *p*-coumaric acid and ferulic acid. Ferulic acid decarboxylation by *B. pumilus* DSM 361 has been demonstrated by Arfmann and Abraham, 1989. These and other authors showed the same activity

in several other microorganisms (Nazareth and Marvinkurve,1986; Huang *et al.*, 1993). However, this activity was further investigated in the rumen isolated *B. pumilus* strain to demonstrate its inducibility and confirm its ability to work in aerobiosis as well as in anaerobiosis. Further work was made in our laboratory to isolate and characterize the enzyme.

B. pumilus is known to be able to produce endoxylanases and β–xylosidases (Panbangred *et al*, 1983; Panbangred *et al.*, 1984). Therefore it is able to utilize xylan, which is the main component of hemicellulose. 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. This is the case of *B. pumilus*: it does not utilize ferulic acid as sole carbon source, but its decarboxylase activity could be a detoxyfication mechanism if 4-vinyl guaiacol was better tolerated by this bacterium, or just a step of a slow complete degradation pathway.

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 highly substrate specificity, but it could be critical in one catabolic pathway leading to specific ring-fission substrates such as protocatechuate and catechol.

B. pumilus has been widely studied for its ability to produce surfactin and to digest xylan. This microorganism has been mutagenized and transformed by electroporation to identify a gene involved in surfactin production (Morikawa *et al.*, 1992). *B. pumilus* genomic DNA digested with PstI restriction enzyme has been inserted in the PstI site of pBR322 and cloned in *E. coli* C600 to identify the genes encoding the enzymes involved in xylan digestion (Panbangred *et al.*, 1983). We wanted to use a similar approach to identify the gene coding for *B.*

pumilus ferulic decarboxylase. For this purpose a *B. pumilus* genomic DNA library was constructed cloning its partially digested DNA in a vector, as suggested by Ausubel *et al.*, 1987. pRS426 and pBR322 plasmids were used as vectors instead of cosmids or phages, which are usually employed to clone large DNA fragments. These vectors offer the advantage of a reduced number of colonies or plaques to screen, but they require a following subcloning pRS426 was initially used because it works as a shuttle and provides a rapid visive screening. The false positive results obtained with this plasmid, cloning 10,000 bp DNA fragments of Sau3AI digested *B. pumilus* DNA, suggested to use the better known and widely employed pBR322 plasmid.

The average size of the fragments to clone was chosen to have a good representation of *B. pumilus* genomic DNA in a limited number of colonies and a big probability to identify the whole gene in one fragment.

On the other hand the N-terminal aminoacid sequence of the ferulic decarboxylase was not yet available and this did not allow to construct a sub-genomic library.

Totally digested DNA was not used to avoid to cut within the gene reducing the ability to identify the intact coding sequence directly with the oligonucleotide probe. Moreover, lacking a direct test to identify positive clones, a large number of colonies should have been checked by hybridization.

Recently a short N-terminal sequence of the protein has been determined allowing to construct four oligonucleotides to be used as probes. However, before using an oligonucleotide to screen a library, it is advisable to perform a series of trial experiments, in which genomic Southern hybridizations are carried out under different degrees of stringency. At first hybridization was carried out, as suggested by Sambrook *et al*, 1989, at 15 °C below the calculated melting temperature ranging between 57-62°C for the four oligonucleotides. However, no signal was detectable after washing at room temperature. Therefore the conditions were changed and hybridization was performed at room temperature

without adding non homologous DNA to the prehybridization solution. The signal was clear using oligonucleotides 3 and 4. In this way the optimal conditions and probes for detection of complementary sequences have been defined. Next step is the screening of the library and eventually the subcloning of the DNA fragments electroeluted from the portion of the agarose gel containing the band detected with the probe.

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