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Characterization of bacterial pectinolytic strains involved in the water retting process

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Summary

Pectinolytic microorganisms involved in the water retting process were characterized. Cultivable mesophilic anaerobic and aerobic bacteria were isolated from unretted and water-retted material. A total of 104 anaerobic and 23 aerobic pectinolytic strains were identified. Polygalacturonase activity was measured in the supernatant of cell cultures; 24 anaerobic and nine aerobic isolates showed an enzymatic activity higher than the reference strains *Clostridium felsineum* and *Bacillus subtilis* respectively. We performed the first genotypic characterization of the retting microflora by a 16S amplified ribosomal DNA restriction analysis (ARDRA). Anaerobic isolates were divided into five different groups, and the aerobic isolates were clustered into three groups. 84.6% of the anaerobic and 82.6% of the aerobic isolates consisted of two main haplotypes. Partial 16S rRNA gene sequences were determined for 12 strains, representative of each haplotype. All anaerobic strains were assigned to the *Clostridium* genus, whereas the aerobic isolates were assigned to either the *Bacillus* or the *Paenibacillus* genus. Anaerobic isolates with high polygalacturonase (PG) activity belong to two clearly distinct phylogenetic clusters related to *C. acetobutylicum–C. felsineum* and *C. saccharobutylicum* species. Aerobic isolates with high PG activity belong to two clearly distinct phylogenetic clusters related to *B. subtilis*[T] and *B. pumilus*[T].

Introduction

Recently, a renewed interest in natural textile fibres, such as linen and hemp, has occurred in Europe and North America. Flax, the raw material for linen production, and hemp have traditionally been grown for their valuable and versatile high-quality bast fibres. Hemp and flax fibres have similar textile properties such as durability, light resistance, absorbency and dyeability (Hamilton, 1986).

The central step in bast fibre production is retting. During this step, the fibre bundles are loosened from the other stem tissues and divided into bundles by depolymerization of pectic substances (Chesson, 1978; Carpita and Gibeaut, 1993). Pectins are complex heteropolysaccharides mainly formed by chains of partially methylated galacturonic residues.

Traditionally, two retting methods (dew retting and water retting) have been used, both carried out by pectic enzymes secreted by indigenous microflora. The extracellular enzyme polygalacturonase (PG) is the primary retting agent (Chesson, 1978; Akin *et al*., 2001; Zhang *et al*., 2000). In dew retting, straw is spread on the ground, and pectins are attacked by pectinolytic microorganisms, mainly filamentous fungi (Henriksson *et al*., 1997). In tank retting, straw is submerged in large water tanks, where a pectinolytic bacterial community develops (Donaghy *et al*., 1990). A succession of pectinolytic microorganisms has been described during flax water retting: *Bacillus* spp. are dominant from 10 to 40 h after the start of the process, and are succeeded by spore-forming anaerobic *Clostridium* spp. when oxygen concentration in water tanks becomes lower. Clostridia are considered to be the major group of bacteria responsible for water retting (Sharma *et al*., 1992; Sharma and Van Sumere, 1992).

The retting process is the major limitation to efficient and high-quality fibre production, thus being the key feature in any future expansion of these industrial crops (Pallesan, 1996). The industrial retting process needs to be enhanced by speeding up the process, improving fibre quality and reducing production costs.

Analysis of the biodiversity of the pectinolytic microflora involved in water retting is important to understand the process dynamics and to improve production. As microorganisms are the main pectinolytic agents during retting, their properties affect the course of the process and end-product quality. Most published research has focused on pectinolytic fungi, involved in dew retting (Henriksson *et al*., 1997). Dew retting is used mainly in northern Europe, but is not suitable for the Mediterranean climate.

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Furthermore, the water process produces more uniform retting and a better quality end-product than dew retting (Akin *et al*., 1998). Recently, PG-producing *Bacillus* strains have been isolated from different sources (Kapoor *et al*., 2000; Kobayashi *et al*., 2001; Sawada *et al*., 2001) and used for retting of fibre crops (Kapoor *et al*., 2001). However, little else is known about pectinolytic bacteria involved in tank retting and their enzymatic properties.

The aim of this work was to improve our knowledge about the microflora involved in the water retting process and to isolate strains with high retting activities. Our strategy involved the analysis of cultivable pectinolytic mesophilic bacteria isolated from hemp or flax sources and the selection of aerobic and anaerobic strains with high PG activity. We performed the first genotypic characterization of the retting microflora using a well-established method of analysis of bacterial communities in different environments (Picard *et al*., 2000; Pukall *et al*., 2001), that is grouping of strains by 16S ribosomal DNA (rDNA) restriction analysis (ARDRA; Vaneechoutte *et al*., 1992) and sequencing the 16S rDNA of at least one member from each ARDRA group.

Results and discussion

Isolation of pectinolytic aerobic and anaerobic strains

Samples were collected from flax and hemp retting water, retted and unretted straw. A total of 225 colonies of spore-forming, mesophilic, anaerobic bacteria were chosen randomly and screened for pectinolytic activity on solid medium in a CO_2 atmosphere. A total of 104 pectinolytic bacteria were isolated. Pectinolytic isolates were recovered from all samples; however, the incidence of pectinolytic isolates was variable. The lowest number of pectinolytic isolates was found in unretted fragments (18.3%); higher proportions were found in water-retted hemp or flax fragments (44.7%) and retting water (100.0% from flax retting liquor and 56.5% from hemp retting liquor).

Aerobic bacteria were isolated from hemp water tanks. A total of 142 colonies, grown under aerobic conditions, were chosen randomly and analysed for pectinolytic activity at 30°C. Only 23 pectinolytic, aerobic, spore-forming, mesophilic bacteria were isolated, and the incidence of pectinolytic isolates was 16.2%.

ARDRA

So far, characterization of pectinolytic microorganisms isolated from flax water retting tanks has been performed by phenotypic analysis, which is often equivocal. For instance, molecular data reveal a phylogenetic incoherence in the genus *Clostridium* (Collins *et al*., 1994). In fact, many of the phenotypic properties traditionally used in the classification of clostridia are not apt to reflect their high degree of genomic unrelatedness and their phylogenetic separateness (Stackebrandt *et al*., 1999). The genetic diversity of the pectinolytic isolates was investigated by a molecular approach based on 16S rDNA analysis (ARDRA), a method that often generates species-specific restriction groups (Grifoni *et al*., 1995; Heyndrickx *et al*., 1996).

DNA was extracted from all the pectinolytic isolates, plus three reference strains of *C. felsineum* and a reference strain of *B. subtilis*, and the 16S rDNA was amplified. ARDRA was performed using three different restriction enzymes (*Alu*I, *Hin*fI and *Rsa*I).

Comparing the restriction patterns of the 104 pectinolytic anaerobic isolates, five different ARDRA haplotypes were recognized (Table 1). Haplotype C was dominant, including 84.6% of the isolates; moreover, this haplotype was found in all the samples analysed. The reference strains of *C. felsineum* (DSM 794^T, NCIMB 10690^T and NCIMB 9539) had the ARDRA haplotype A. Isolates with haplotype A were isolated from both retted fragments and tank water samples. The other haplotype groups (B, D and E) were smaller, formed by less than five isolates each, and each pattern was found in a single sample only, water hemp tanks, unretted fragments and retted fragments respectively.

Three different haplotypes were defined by analysing the restriction patterns of the pectinolytic aerobic isolates (Table 2). 82.6% of the isolates had the major haplotype F. The other haplotypes (G and H) were found in less than four isolates. The reference strain *B. subtilis* 168 had the haplotype G.

16S rDNA sequencing and phylogenetic analysis

The almost complete sequence of the 16S rDNA of at least one strain from each ARDRA haplotype was determined. Sequences were compared with databases, and their best matches are shown in Table 3. The 16S rDNA of isolates and the retrieved sequences were used to construct phylogenetic trees (Fig. 1).

Sequence analysis confirmed that all the anaerobic, spore-forming pectinolytic strains are members of four clearly distinct groups, all included in the genus *Clostridium* (Fig. 1A). 16S rDNA sequence analysis and estimation of phylogenetic relationships assigned all the strains to *Clostridium* group I; this phylogenetic clade was proposed by Collins *et al*. (1994) as the base for a redefined genus *Clostridium*.

As shown by the ARDRA data, strains belonging to the haplotypes A and E have a sequence similarity >99.0% with both *C. felsineum*^{T} and *C. acetobutylicum*^{T}, two species phylogenetically closely related (Tamburini *et al*., 2001).

Table 1. PG activity of anaerobic strains and ARDRA haplotypes.

			Distribution into ARDRA haplotypes				
Strain name[a]	No. of strains	PG activity[b]	A	B	C	D	E
C1/5, C3/5, F/1, C1/6, C3/6, L1/6, FA3/2	7	100.00–200.00	6				1
L1/5, F7/4, F7/7, FA3/1, L1T/2, L1/8, C3T/2, C3/1, C2T/3, L1T/3, F7/6, C3T/10, C1T/8, L1/12, L1/9, C1T/10, L1T/5	17	31.00–99.00	1		15		1
C4T/6, C3T/3, C3T/5, L1T/11, C1T/13, C3/3, F8/9, C3T/1, C1T/2, C1T/9, L1T/1, F7/2, C1T/14, F7/3, L1T/12, L1T/4, F7/9, C4T/1, F8/1, C4T/7, C3T/6	21	20.00–30.00			21		
F2II/10, C1T/4, L1T/7, L1T/8, C3T/8, C3/2, C4T/4B, C1T/5, C3T/14, FA2/18, C1T/15, C1T/11, C1T/12, L1/1, L1T/6, F7/1, FA2/16, C1T/6, C3T/12, L1/7, FA1/10, C3T/9	22	10.00–19.00			19	3	
C4T/10, F2II/5, C3T/13, F8/5, C1T/1, F8/2, C3T/7, F2II/9, F8/3, C3T/15, F8/7, F7/10, F2II/6, C1T/7, C3T/11, F2II/4, F2II/3, C3T/4, F2II/1, F8/8, C4T/15, C4/1, L1T/9, FA2/6, L1/2, C4/2, F2II/2, F8/4, L1/3, C3/4, C4T/13, F2II/7, F7/5, F7/8, L1T/10, C1T/3, L1/10	37	<10.00	1	2	33	1	

a. The first letter of the name identifies the origin of the strain: C, water from hemp retting tanks; L, water from flax retting tanks; F, retted or unretted hemp or flax fragments.
b. Expressed as IU g^{-1} cells wet weight. *C. felsineum* reference strains had an activity of 30.00 IU g^{-1} cells wet weight.

These species have been described previously as the anaerobic agents of water retting (Donaghy *et al*., 1990).

The four members of the major haplotype C (L1/8, F7/4; F7/7 and C1T/10) were grouped in a single cluster with *C. saccharobutylicum*^{T} (99.9–100.0% sequence similarity) (Keis *et al*., 1995; 2001). These data suggest that the haplotype C group is a rather homogeneous cluster. Interestingly, *C. saccharobutylicum* has never been described as being involved in water retting. However, several strains previously classified as *C. acetobutylicum* on the basis of phenotypic traits have been assigned to this new species, recently defined by 16S rDNA sequencing and DNA–DNA reassociation analysis (Keis *et al*., 2001).

Sequence analysis of the 16S rDNA of C4/1 isolate (haplotype B) retrieved sequences with low similarity values and may represent a novel species, phylogenetically related (95.6% similarity) to *Anaerobacter polyendosporus* (Siunov *et al*., 1999). This polysporogenic species should probably be reclassified as a member of *Clostridium* on the basis of 16S rDNA similarity with cluster I members (Stackebrandt *et al*., 1999). Phylogenetic analysis showed the 16S rDNA sequence of strain FA2/18 (haplotype D) is most closely related to *C. aurantibutyricum*^{T}.

As suggested by ARDRA, aerobic strain ROO2A (haplotype G) was assigned to the *B. subtilis*^{T} cluster (98.3–99.9% sequence similarity; Nakamura *et al*., 1999) (Fig. 1B). Analysis of 16S rDNA of strain ROO40B (major haplotype F) showed 100% similarity to 16S rDNA sequences of two uncharacterized marine epiphytic strains and was placed in the *B. pumilus*^{T} cluster (99.4%-100% sequence similarity). This analysis confirms the numerical dominance of *Bacillus* spp. in the aerobic step of water retting.

Table 2. PG activity of aerobic strains and ARDRA haplotypes.

			Distribution into ARDRA haplotypes		
Strain name	No. of strains	PG activity[a]	F	G	H
ROO40B, ROO2A	2	100.00–200.00	1	1	
ROO71AI, ROO35BI, ROO71B, ROO9A, ROO71AII, ROO72B, ROO37A	7	40.00–99.00	5	2	
ROO43A, ROO35BII, ROO31A, UNO51AI, ROO62A, ROO14A, UNO51AII, ROO78BI, ROO66B, ROO66A, ROO9B	11	20.00–39.00	11		
ROO62B	1	10.00–19.00	1		
ROO37B, ROO32A,	2	<10.00	1		1

a. Expressed as IU g^{-1} cells wet weight. *B. subtilis* reference strain had an activity of 39.00 IU g^{-1} cells wet weight.

Table 3. Sequence similarity between the 16S rRNA gene of pectinolytic strains and the best match in databases.

	ARDRA haplotype	Best match[a]	Sequence similarity (%)[b]
Anaerobic strains			
C1/6	A	*Clostridium felsineum* DSM 794^T (AF270502)	99.9
L1/6	A	*Clostridium felsineum* DSM 794^T (AF270502)	99.9
C4/1	B	Anaerobacter polyendosporus (AJ222546)	95.6
F7/4	C	*Clostridium saccharobutylicum* DSM 13864^T (U16147)	100.0
F7/7	C	*Clostridium saccharobutylicum* DSM 13864^T (U16147)	99.9
C1T/10	C	*Clostridium saccharobutylicum* DSM 13864^T (U16147)	99.9
L1/8	C	*Clostridium saccharobutylicum* DSM 13864^T (U16147)	99.9
FA2/18	D	*Clostridium aurantibutyricum* NCIMB 10659^T (X68183)	99.8
FA3/2	E	*Clostridium acetobutylicum* ATCC 824^T (U16166)	99.0
Aerobic strains			
ROO2A	G	*Bacillus subtilis* ssp. *spizizenii* NRRL B-23049 (AF074970)	99.9
ROO40B	F	EI-44-7 (AJ494734) and EI-26-7 (AJ494730) isolates	100.0
ROO32A	H	*Paenibacillus amylolyticus* NRRL NRS-290^T (D85396)	98.8

a. GenBank accession numbers are shown in parenthesis.
b. Similarity values were calculated in the region of overlapping and no gaps – insertions or deletions – were included in the match/mismatch count in all the analysis. Ambiguity codes are matched correctly.

The only member of haplotype H, strain ROO32A, was assigned to the *Paenibacillus* genus by sequence analysis and by estimation of phylogenetic relationships (Fig. 1B), showing highest similarity to *P. amylolyticus*^{T}. ROO32A is the first strain of this genus isolated from tank retting material. Interestingly, an endophytic bacterium, tentatively identified as *P. amylolyticus*, has been described as producing pectin lyase activity (Sakiyama *et al*., 2001).

PG activity of pectinolytic aerobic and anaerobic strains

The PG activity of all the pectinolytic strains was measured in the supernatant of liquid cultures and compared with the activity of the reference strains. We determined this enzymatic activity because PG is considered to be the most important enzyme in the retting process (Zhang *et al*., 2000; Akin *et al*., 2001). The activity levels of anaerobic strains covered a wide range. Twenty-four anaerobic isolates showed a PG activity higher than *C. felsineum* reference strains (> 31.00 IU g^{-1} cells) and, among them, seven showed an activity >100.00 IU g^{-1} cells (Table 1). These 24 strains were isolated from flax retting tanks (33.3%), retted flax or hemp fragments (25.0%) and hemp retting tanks (41.7%). Unretted straw is the least suitable material for isolation of strains with high activity, as shown by the lower proportion of pectinolytic isolates and their enzymatic properties. The most active strains were assigned to haplotypes A, E and C. Strains belonging to the major haplotype C group present a wide range of PG activities. This haplotype includes a large proportion of isolates with high PG activity and most of the pectinolytic bacteria isolated in this study. All the strains with either haplotype B or D showed PG activity <19.00 IU g^{-1} cells.

Eleven anaerobic strains with high PG activity were used previously in laboratory tests of hemp water retting (Di Candilo *et al*., 2000). Inoculation with spores of L1/6 and C1/6, assigned to the *C. felsineum*^{T} and *C. acetobutylicum*^{T} groups, gave the best results, significantly reduced the retting time and produced a softer and finer fibre. Results that fell halfway between the two mentioned above and the control (which required 12 days of retting) were obtained with three isolates (C1/5, C3/5, F/1) of haplotype A and isolate FA3/2. Similar results were also obtained with strains L1/8, F7/7 and F7/4, assigned to the *C. saccharobutylicum*^{T} cluster, and with two isolates (L1/5, L1T/2) of haplotype C.

When PG activity was measured under the conditions used for anaerobic bacteria (citrate buffer pH 4.8, 45°C), none of the aerobic pectinolytic isolates showed detectable activity. The same result was obtained at different incubation temperatures (32°C, 37°C, 40°C). On the other hand, most of the isolates showed detectable PG activity under the experimental conditions described previously by Kobayashi *et al*. (2001), Tris-HCl buffer, pH 8.0, and 30°C (Table 2). Nine aerobic isolates showed PG activity higher than the reference strain *B. subtilis* 168 (>39.00 IU g^{-1} cells). Two strains showed activity higher than 100.00 IU g^{-1} cells. All strains with high PG activity were assigned to either the major ARDRA haplotype F or the minor haplotype G, whereas the isolate with haplotype H showed very low PG activity.

One of the aerobic strains with the highest PG activity (ROO2A), assigned to the *B. subtilis*^{T} cluster, was used in laboratory tests of hemp water retting. This preliminary test showed that the shortest retting time (4 days) was obtained when tanks were simultaneously inoculated with both aerobic and anaerobic bacteria (data not shown). Inoculation with only the aerobic strain did not reduce the retting time, and the fibre was of poor quality.

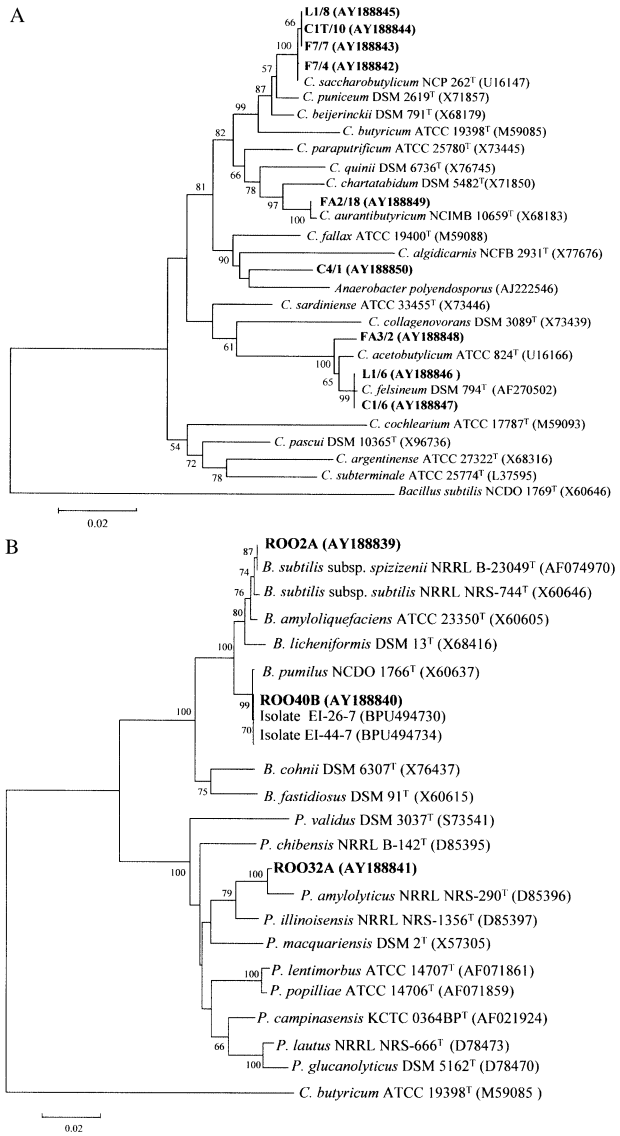

Fig. 1. Unrooted phylogenetic tree based on 16S rDNA comparisons showing the position of pectinolytic isolates and related species. Bootstrap probability values that were <50% were omitted. The scale bar indicates substitutions per nucleotide position. The GenBank accession numbers for the 16S rRNA sequences are reported after the strain number. Relationships of (A) anaerobic isolates and *Clostridium* spp. (data set including 125–1452 nt *E. coli* numbering); (B) aerobic isolates and *Bacillus* spp., *Paenibacillus* spp. (data set including 101–1418 nt *E. coli* numbering).

Conclusion

In conclusion, our data show that the isolated anaerobic and aerobic bacteria have heterogeneous PG activities and that the natural population is a source of genetic biodiversity, which can be used to speed up the water retting process and to increase end-product quality.

An inoculum of anaerobic PG-producing bacteria can significantly reduce the retting time and improve fibre quality. Furthermore, measurement of PG activities allows the selection of bacteria with good retting properties. In the retting process, spore germination of anaerobic bacteria requires a reduction in oxygen content. This is accomplished by the growth of aerobic bacteria. Even if anaerobic bacteria seem to be the main retting microorganisms (Sharma *et al.*, 1992; Sharma and Van Sumere, 1992), the biodiversity of pectinolytic aerobic bacteria isolated from hemp tanks and their PG activities were evaluated. However, none of the pectinolytic isolates showed PG activity at pH 4.8, which is close to that of the ret liquor (Chesson, 1978; Donaghy *et al.*, 1990). This suggests that PG enzymes produced by aerobic bacteria could play a minor role in the retting process, except in the early stages before the ret liquor becomes acid. On the other hand, the aerobic strains showed PG activity at pH 8.0. One of the aerobic strains with the highest PG activity was used in laboratory tests of hemp water retting. This preliminary test showed that the shortest retting time (4 days) was obtained when tanks were simultaneously inoculated with both aerobic and anaerobic bacteria.

High PG-producing strains isolated in this work could be useful for isolation of new biotechnologically important enzymes. In particular, few clostridial pectinolytic enzymes have been characterized to date (Lee *et al.*, 1970; Van Rijssel *et al.*, 1993; Tamaru and Doi, 2001). These enzymes are important not only in the textile industry but also in papermaking and extraction, clarification and liquefaction of fruit juices and wine (Kashyap *et al.*, 2001).

Experimental procedures

Isolation procedures, culture conditions and reference strains

Bacterial strains were isolated from the liquor of hemp or flax water retting tanks. Samples of unretted and water-retted flax or hemp fragments were also used. Straw was washed with sterile distilled water. Ret liquor and straw washing water were heated at 80°C for 5 min to select spore-forming bacteria. Aerobic bacteria were grown on agar plates of rich medium (0.5% yeast extract, 0.5% pectone, 1% tryptone) at 30°C; anaerobic bacteria were grown on the same medium supplemented with 2% glucose and 0.05% cysteine at 37°C in a CO_2 atmosphere (Oxoid AnaeroGen kit).

Clostridium felsineum strains DSM 794^T, NCIMB 10690^T and NCIMB 9539 were used as reference strains for anaerobic isolates, whereas *B. subtilis* 168 was used as reference for aerobic isolates.

Enzymatic assays

To identify pectinolytic strains, randomly chosen colonies were transferred onto solid rich medium supplemented with 0.5% pectin from citrus peel (Sigma). After growth, plates

were flooded with a 1% solution of cetyltrimethyl ammonium bromide (Donaghy *et al*., 1990). Clearing around colonies indicated pectinolytic activity.

PG activity was measured in the supernatants of cell cultures by the DNS (dinitrosalicylic acid) method (Miller, 1959). Aerobic strains were grown for 2 days at 30°C with shaking (250 r.p.m.), whereas anaerobic strains were grown for 3 days at 37°C in a CO_2 atmosphere in rich medium supplemented with 0.5% pectin. For anaerobic strains, PG activity was tested in a reaction mixture (1 ml volume) containing 50 mM citrate buffer, pH 4.8, and 0.9% (w/v) polygalacturonic acid (Sigma). After incubation for 20 min at 45°C, the reaction was terminated by adding 3 ml of DNS and 1 ml of water, followed by heating at 100°C for 10 min. The suspension was centrifuged, and the supernatant OD was measured at 640 nm. PG activity of aerobic strains was tested by the DNS method as described by Kobayashi *et al*. (2001).

Enzyme activity was determined by reference to standard galacturonic acid (Sigma) solutions. One unit (IU) corresponds to the release of 1 μmol of reducing groups (final products of the reaction) in 1 min at the assay temperature by 1 ml of supernatant. The IU were corrected by the culture wet weight (IU g^{-1} cells). Activities were determined in two separate experiments with two independent measurements each.

DNA extraction and 16S rDNA polymerase chain reaction (PCR) amplification

Well- isolated colonies were picked up from an agar plate of rich medium with a sterile toothpick and resuspended in 200 μl of sterile water. DNA was extracted using the FastDNA kit in a FastPrep Instrument, according to the manufacturer's specifications (BIO101; Quantum Technologies). The 16S rDNA amplification was performed using primers P0 and P6 designed on the basis of conserved bacterial sequences at the 5′ and 3′ ends of the 16S rDNA (Picard *et al*., 2000). The PCR volume was 20 μl, containing 2 μl of sample, 1 unit of *Taq* DNA polymerase (Life Technologies), 250 μM each dNTP, 1 μM each PCR primer, 1.5 mM $MgCl_2$ and 2 μl of 10× PCR buffer (Life Technologies). A 35-cycle touch-down PCR programme (30 s denaturation at 95°C, 30 s annealing at temperatures decreasing by 5°C from 60°C to 50°C every five cycles, 2 min elongation at 72°C and 10 min final elongation step at 72°C) was performed using GenAmp PCR system 9600 (Perkin-Elmer). An aliquot of 2 μl of each reaction mixture was analysed by agarose (0.7% w/v) gel electrophoresis in TAE buffer containing 0.5 μg ml^{-1} ethidium bromide.

ARDRA

Aliquots of 3–5 μl of the amplified DNA, corresponding to ≈1.5 μg, were completely digested according to the manufacturer's specifications for 3 h with 3 units of the restriction endonucleases *Alu*I, *Rsa*I and *Hin*fI (Life Technologies). The restriction products were electrophoresed on a 2.5% (w/v) agarose gel in TAE buffer containing 0.5 μg ml^{-1} ethidium bromide. The 100 bp DNA Ladder (Life Technologies) was used as a molecular size standard.

16S rDNA sequencing and phylogenetic analysis

The amplified 16S rDNA was purified from the PCR mixture with the High Pure PCR product purification kit (Roche). The determination of 16S rDNA nucleotide sequences was performed with a Perkin-Elmer ABI 310 sequence analyser.

For phylogenetic analysis, the 16S rDNA sequences were compared with the prokaryotic small subunit rRNA sequence database of the Ribosomal Database Project II (RDP) (Maidak *et al*., 2001), by RDP utilities, and with GenBank by the BLASTN program (Altschul *et al*., 1990). 16S rDNA sequences of isolates and related sequences, retrieved from databases, were aligned with the MULTALIN software (Corpet, 1988). The resulting alignments were checked manually and corrected if necessary. Phylogenetic trees were inferred using the neighbour-joining method (Saitou and Nei, 1987). An evolutionary distance matrix was generated as described by Jukes and Cantor (1969). The software MEGA, version 2.0, was used to construct trees (Kumar *et al*., 2001). Bootstrap analysis (1000 replicates) was used to test the topology of the neighbour-joining method data.

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