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The acetyl xylan esterase of *Bacillus pumilus* belongs to a family of esterases with broad substrate specificity

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The *Bacillus pumilus* gene encoding acetyl xylan esterase (*axe*) was identified and characterized. The *axe* gene was expressed and the recombinant enzyme produced in *Escherichia coli* was purified and characterized. The recombinant enzyme displayed similar properties to the acetyl xylan esterase (AXE) purified from *B. pumilus*. The AXE primary structure was 76% identical to the cephalosporin C deacetylase of *B. subtilis*, and 40% to two recently identified AXEs from *Thermoanaerobacterium* and *Thermotoga maritima*. These four proteins are of similar size and represent a new family of esterases having a broad substrate specificity. The recombinant AXE was demonstrated to have activity on several acetylated substrates, including on cephalosporin C.

Keywords: *Bacillus pumilus*, acetyl xylan esterase, cephalosporin C deacetylase

INTRODUCTION

Biodegradation of hemicellulose is a process that requires the cooperation of several enzymes. Xylan is the major constituent of hemicellulose and after cellulose it is the most abundant renewable polysaccharide in plants; several bacteria and fungi grow on xylan as a carbon source by using an array of enzymes, such as endoxylanases and β-xylosidases. Xylan of hemicellulosic polysaccharide plant cell walls is predominantly a 1,4-β-D-xylose polymer and is commonly substituted to various degrees with acetyl, arabinosyl and glucuronyl residues (Whistler & Richards, 1970). This structural complexity thus requires the cooperation of xylanases and β-xylosidases with several accessory enzymes for its biodegradation. About 60–70% of xylose residues are esterified at the hydroxyl group with acetic acid; these acetylated xylans are abundant in hardwood (Lindberg *et al*., 1973). The existence of acetyl xylan esterases (AXEs) was first reported in fungal cultures (Biely, 1985; Biely *et al*., 1986), and also the synergistic activity of partially purified AXEs of *Schizophyllum commune* with xylanases, with production of xylo-oligomers, xylose and acetic acid. Following these reports, a number of enzymes with the same activity have been described from both fungi and bacteria, and have been reviewed by Christov & Prior (1993). AXEs (EC 3.1.1.72) are enzymes that hydrolyse the ester linkages of the acetyl groups in position 2 and/or 3 of the xylose moieties of natural acetylated xylan from hardwood. These enzymes are one of the accessory enzymes which are part of the xylanolytic system, together with xylanases, β-xylosidases, α-arabinofuranosidases and methylglucuronidases; these are all required for the complete hydrolysis of xylan (Biely, 1985).

The xylanolytic system of *Bacillus pumilus* has been investigated in part and genes encoding xylanase and β-xylosidase enzymes have been identified and cloned (Moriyama *et al*., 1987; Panbangred *et al*., 1984). We have recently reported for the first time the purification and characterization of an inducible and secreted AXE from *B. pumilus* PS213 (Degrassi *et al*., 1998). We report here the cloning of the acetyl xylan esterase gene (*axe*) of *B. pumilus*, and its expression and purification in *Escherichia coli*. The recombinant protein had similar properties to the purified AXE of *B. pumilus* and belongs to the esterase family 7 (Coutinho & Henrissat, 1999). Interestingly, the highest identity (76%) of the *B. pumilus* AXE was observed with the cephalosporin C deacetylase of *B. subtilis*. The expression of the *B. pumilus axe* gene in *E. coli* resulted in high-level production of the enzyme. Finally, the recombinant *B. pumilus* AXE (rAXE) had activity on several acetylated substrates including cephalosporin C; esterase enzymes of this family possess broad substrate specificity.

Abbreviations: AXE, acetyl xylan esterase; rAXE, recombinant xylan esterase.

The GenBank/EMBL/DDBJ accession number for the sequence reported in this paper is AJ249957.

METHODS

Bacterial strains, plasmids and culture conditions. *E. coli* DH5α (Hanahan, 1983) and XL-1 Blue (Bullock *et al*., 1987) were used (Table 1). Growth was at 37 °C in Luria–Bertani (LB) broth supplemented when necessary with 100 μg ampicillin ml^{-1}, 0·2 mM IPTG and 20 μg X-Gal ml^{-1}. *B. pumilus* PS213 is an aerobic soil bacterium, initially isolated from rumen fluid; it was grown at 30 °C in LB medium. Plasmids used are listed in Table 1.

Isolation of *B. pumilus axe* gene and recombinant DNA techniques. Two degenerate oligonucleotide probes were synthesized and used for the PCR amplification of a region of the *axe* gene of *B. pumilus*. The *B. pumilus* genomic DNA was prepared according to Ausubel *et al*. (1988) and used as a template for PCR. The oligonucleotide primers were an 18-mer called BPEN [5′-ATGCA(AG)(CT)T(ACGT)TT(CT)-GA(CT)(CT)T(ACGT)-3′] and a 21-mer designated BPEI [5′-(CT)TC(AG)TC(ACGT)AC(CT)TC(ACGT)GG(AG)-AA(ACGT) (GC) (AT)-3′]. Digestion with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, ligation with T4 DNA ligase, end-filling with Klenow fragment of DNA polymerase, hybridizations, radioactive labelling by random priming and transformation of *E. coli* were performed as described by Maniatis *et al*. (1982). Southern hybridizations were performed using Hybond-N+ membrane (Amersham Phamacia Biotech). Plasmids were purified as described by Birnboim (1983) and with Qiagen columns.

DNA sequence determination and analysis. DNA fragments from plasmid pBPEB2 harbouring the 2·3 kb fragment containing the *axe* gene were prepared by digestion with *Hin*dIII and *Xba*I and cloned directly in pBluescript II SK(+) and pBluescript II KS(+). The constructs were either encapsidated as single-stranded DNA upon infection with helper phage VCSM13 (Stratagene) or used directly for DNA sequencing. Fifteen oligonucleotides (17-mers) were synthesized and used as primers in the sequencing reactions. DNA sequences were determined by the dideoxy chain-termination method of Sanger *et al*. (1977), using [^{35}S]dATPαS for labelling.

Enzyme assay and purification. AXE was routinely assayed by measuring the conversion of 2 mM α-naphthyl acetate to α-naphthol at 37 °C for 10 min according to Poutanen & Sundberg (1988), except that the pH of the reaction mixture was 7·0. One unit of activity was defined as the amount of enzyme required to produce 1 μmol product min^{-1} under the assay conditions. To determine the substrate specificity of rAXE, the deacetylation of *p*-nitrophenyl acetate, 4-methyl-umbelliferyl acetate, xylose tetraacetate and glucose penta-acetate (Sigma and see Table 3) was also determined, following the procedures previously described (Degrassi *et al*., 1998).

Acetyl esterase activity was also determined *in situ* after IEF, by spreading 2 mM α-naphthyl acetate onto the gel, incubating for 10 min at 37 °C and then adding 5 % Fast Garnet GBC in 10 % SDS. Optimal pH and temperature for the rAXE were determined in the range pH 3–9·5 (50 mM sodium acetate, pH 3·0–5·5; sodium phosphate, pH 6·0–7·0; Tris/HCl, pH 7·5–9·5) and 4–80 °C, respectively. In both cases determination was made using 2 mM α-naphthyl acetate in 10 min assays at 37 °C. For optimal temperature determination, 50 mM phosphate buffer, pH 7·0, was used.

rAXE was purified to homogeneity from the supernatant of 1 l recombinant *E. coli* culture, grown on LB medium overnight at 37 °C on a rotary shaker. Chromatography was performed at room temperature using a low-pressure liquid chromatography system (GradiFrac; Pharmacia Biotech), following the procedure adopted for the purification of the AXE from *B. pumilus* and previously described (Degrassi *et al*., 1998), except that the first step (Q Sepharose column) was omitted.

Protein analysis. After gel filtration chromatography, the purified protein was analysed by SDS-PAGE (12 % acrylamide). Molecular mass markers were from Amersham Life Science, as follows: myosin, 220 kDa; phosphorylase *b*, 97·4 kDa; albumin, 66 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20·1 kDa; α-lactalbumin, 14·4 kDa.

The pI of the rAXE was determined by using an Ampholine PAGplate precast polyacrylamide gel (Pharmacia Biotech), with pH values ranging from 3 to 10, and by using the broad-pI calibration kit (Pharmacia Biotech) as the pI marker, according to the instructions of the supplier. Protein bands in SDS-PAGE and IEF gels were stained with Coomassie blue R-250, according to Sambrook *et al*. (1989).

Other enzyme assays. Cephalosporin C deacetylase activity of the rAXE was assayed in 50 mM sodium phosphate buffer (pH

Table 1. Strains and plasmids used

Ap^r,Tc^r: resistant to ampicillin and tetracycline, respectively.

Strain or plasmid	Relevant characteristic	Reference
Strains		
E. coli DH5α	Δ*lacU169*(ϕ80 *lacZ*ΔM15)	Hanahan (1983)
E. coli XLIBMRF	F′, Tn*10* (Tc^r)	Bullock *et al*. (1987)
B. pumilus PS213	Wild-type	Degrassi *et al*. (1998)
Plasmids		
pUC18	Ap^r, ColE1 replicon	Yanisch-Perron *et al*. (1985)
pBluescript II SK(+)	Ap^r, ColE1 replicon	Stratagene
pBluescript II KS(+)	Ap^r, ColE1 replicon	Stratagene
pMPAX	pUC18 with 516 bp of *B. pumilus* DNA	This study
pBPE2	pBluescript II SK(+) with 2·26 kb *Eco*RI fragment of *B. pumilus* DNA	This study

7·0) at 37 °C for 30 min and measured according to the procedure of Takimoto *et al*. (1994). The reaction mixture was analysed by reverse-phase HPLC using a RP-C18 column (5 mm, 15 × 0·4 cm) and 90% 43 mM potassium phosphate buffer, pH 5·2/10% methanol as eluent. Flow rate was 0·8 ml min^{-1} and peaks were monitored at 254 nm. To determine the Michaelis constants, the reaction was performed as described above with concentration of cephalosporin C (Sigma) or 7-aminocephalosporanic acid (7-ACA; Sigma) ranging from 0·05 to 5 mM.

RESULTS

Cloning of the acetyl xylan esterase gene (*axe*) of *B. pumilus*

The AXE enzyme of *B. pumilus* was previously purified to homogeneity and was subjected to N-terminal amino acid sequencing; in addition, the amino acid sequence of an internal fragment was also determined following trypsin digestion (Degrassi *et al*., 1998). On the basis of these two amino acid sequences, two degenerate oligonucleotides were synthesized and used in a PCR reaction using *B. pumilus* genomic DNA as template (see Methods); the BPEN oligonucletide was designed based on the N-terminal sequence (MQLFDLFLEELG; Degrassi *et al*., 1998), whereas BPEI was designed from an internal amino acid sequence (ALEVIQSFPEV-DEHR, Degrassi *et al*., 1998). The product of the reaction was a DNA fragment of approximately 516 bp. This fragment was cloned in pUC18, giving rise to pMPAX. The sequence of this fragment confirmed the presence of both oligonucleotides used in the PCR reaction, and furthermore displayed significant high homology with the *axe* gene of *Thermoanaerobacterium* and of the cephalosporin C deacetylase of *B. subtilis*. It was concluded that the amplified DNA fragment was

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B. pumilus                MQLFDLSLEELKKYKPKKTARPDFSDFWKKSLEELRQVEAEPTLESYDYP 50
B. subtilis               MQLFDLPLDQLQTYKPEKTAPKDFSEFWKLSLEELAKVQAEPDLQPVDYP 50
Thermotoga maritima       MAFFDLPLEELKKYRPERYEEKDFDEFWEETLAESEKFPLDPVFERMESH 50
Thermoanaerobacterium     MGLFDMPLQKLREYTGTNPCPEDFDEYWNRALDEMRSVDPKIELKESSFQ 50
                          * :**:.*::*: *   .    **.::*: :* *  ..  .  ::  .

B. pumilus                VKGVKVYRLTYQSFGHSKIEGFYAVPDQTGPHPALVRFHGYNASYDGGIH 100
B. subtilis               ADGVKVYRLTYKSFGNARITGWYAVPDKEGPHPAIVKYHGYNASYDGEIH 100
Thermotoga maritima       LKTVEAYDVTFSGYRGQRIKGWLLVPKLEEEKLPCVVQYIGYNGGRGFPH 100
Thermoanaerobacterium     VSFAECYDLYFTGVRGARIHAKYIKPKTEGKHPALIRFHGYSSN-SGDWN 99
                           . .: * : : .    :* .    *.    : . :  :    .  *  :

B. pumilus                DIVNWALHGYATFGMLVRGQGGSEDTSVTPGG-------HALGWMTKGIL 143
B. subtilis               EMVNWALHGYATFGMLVRGQQSSEDTSISPHG-------HALGWMTKGIL 143
Thermotoga maritima       DWLFWPSMGYICFVMDTRGQGSGWLKGDTPDYPEGPVDPQYPGFMTRGIL 150
Thermoanaerobacterium     DKLNYVAAGFTVVAMDVRGQGGQSQDVGGVTG------NTLNGHIIRGLD 143
                          : : :   *:  . * .*** .                    * : :*:

B. pumilus                S-KDTYCYRGVYLDAVRALEVIQSFPEVDEHRIGVIGGSQGGALAIAAAA 192
B. subtilis               D-KDTYYYRGVYLDAVRALEVISSFDEVDETRIGVTGGSQGGGLTIAAAA 192
Thermotoga maritima       D-PRTYYYRRVFTDAVRAVEAAASFPQVDQERIVIAGGSQGGGIALAVSA 199
Thermoanaerobacterium     DDADNMLFRHIFLDTAQLAGIVMNMPEVDEDRVGVMGPSQGGGLSLACAA 193
                          .   .  :* :: *:.:      .: :**: *: : * ****.:::* :*

B. pumilus                LSDIPKVVVADYPYLSNFERAVDVALEQ-PYLEINSYFRRNSDPK-VEEK 240
B. subtilis               LSDIPKAAVADYPYLSNFERAIDVALEQ-PYLEINSFFRRNGSPE-TEVQ 240
Thermotoga maritima       LSKKAKALLCDVPFLCHFRRAVQLVDTH-PYAEITNFLKTHRDK---EEI 245
Thermoanaerobacterium     LEPRVRKVVSEYPFLSDYKRVWDLDLAKNAYQEITDYFRLFDPRHERENE 243
                          *.   :  :.: *:*..:.*. ::   : .* **..:::       *

B. pumilus                AFETLSYFDLINLAGWVKQPTLMAIGLIDKITPPSTVFAAYNHLETDKDL 290
B. subtilis               AMKTLSYFDIMNLADRVKVPVLMSIGLIDKVTPPSTVFAAYNHLETKKEL 290
Thermotoga maritima       VFRTLSYFDGVNFAARAKIPALFSVGLMDNICPPSTVFAAYNYYAGPKEI 295
Thermoanaerobacterium     VFTKLGYIDVKNLAKRIKGDVLMCVGLMDQVCPPSTVFAAYNNIQSKKDI 293
                          .: .*.*:*  *:*   *  .*:.:**:*:: **********     *::

B. pumilus                KVYRYFGHEFIPAFQTEKLSFLQKHLLLST 320
B. subtilis               KVYRYFGHEYIPAFQTEKLAFFKQHLKG-- 318
Thermotoga maritima       RIYPYNNHEGGGSFQAVEQVKFLKKLFEKG 325
Thermoanaerobacterium     KVYPDYGHEPMRGFGDLAMQFMLELYS--- 320
                          ::*   .**   .*       : :
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Fig. 1. Protein sequence alignment using the one letter code of *B. pumilus* AXE, *B. subtilis* cephalosporin C deacetylase (P94388, Q59233), AXE of *Thermotoga maritima* (AAD35171) and the AXE of *Thermoanaerobacterium* sp. JW/SL (O30361). * indicates conserved amino acids; : indicates conserved substitutions; . indicates semi-conserved substitutions. The amino acids which are in the boxed region represent the signature of the esterase common sequence (see text for details).

Table 2. Purification of rAXE from *E. coli* supernatant

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification factor	Yield (%)
Culture supernatant	40	220	5·5	1	100
$(NH_4)_2SO_4$ fractionation	19·2	127	6·6	1·2	57·6
Phenyl Sepharose HR	5·4	104	19·3	3·5	47·3
Q Sepharose Fast Flow	0·72	44·1	61·2	11·1	20
Sephacryl HR200	0·2	28·8	144	26·1	13·1

part of the *axe* gene of *B. pumilus*. To isolate the *axe* gene of *B. pumilus*, genomic DNA was digested with several restriction enzymes and after Southern transfer was hybridized with the PCR fragment as probe and under conditions of high stringency. A 2·3 kb *Eco*RI single discrete band of hybridization was seen (data not shown). The 2·3 kb *Eco*RI hybridizing fragment was then cloned into the *Eco*RI restriction site of pBluescript II SK(+), creating pBPEB2. It was assumed that the clone pBPEB2 contained the gene(s) which were previously amplified by PCR.

Nucleotide sequence of the *B. pumilus axe* gene

To determine the nucleotide sequence of the *B. pumilus axe* gene, different restriction fragments from plasmid pBPEB2 were cloned in pBluescript II SK(+) and KS(+) and sequenced. The 2·26 kb *Eco*RI fragment was sequenced in both directions and the sequence deposited in the GenBank/EMBL/DDBJ database under accession number AJ249957. Nucleotide sequencing of this fragment revealed the presence of an open reading frame starting at nucleotide 841 until 1800 encoding a protein of 320 amino acids with a predicted molecular mass of 35 989 Da. This ORF, representing a gene designated *axe*, was preceded by a potential Shine–Dalgarno sequence (AAAGGGGAA) and a putative transcription termination signal was noted 57 bp after the stop codon (GCTAAAATGA–3bp–TCATTTTAGC). Analysis of the deduced amino acid of the AXE protein revealed striking identity (76%) with the cephalosporin C deacetylase protein isolated from *B. subtilis* (Mitsushima *et al*., 1995). This *B. pumilus* AXE also displayed high identity (42%) with the putative AXE of *Thermotoga maritima* and 38% identity with the AXE of *Thermoanaerobacterium*; in Fig. 1 an alignment of these four very related proteins is shown. All four AXE proteins have an esterase/lipase/thioester common sequence (protein prosite signature PS50187; Fig. 1). In the *B. pumilus* AXE it is located between positions 179 and 184 and is GGSQGG; the motif of this common sequence is GXSXG (Brenner, 1988). The AXE protein contained both the amino acid sequences previously determined (Fig. 1 and Degrassi *et al*., 1998) and, together with the protein similarities with other AXEs, it was therefore concluded that the gene encoding the *B. pumilus* AXE protein was isolated.

Expression, purification and characterization of recombinant AXE

Acetyl esterase activity against α-naphthyl acetate was detected in the supernatant of the *E. coli* culture harbouring pBPEB2. It was observed that this enzyme present in supernatants was due to cell lysis and not as a result of protein secretion. This was verified by the observation via SDS-PAGE that other proteins are also released in the supernatant (data not shown). The specific activity of the supernatant was 5·5 U mg^{-1}, eightfold higher than the 0·66 U mg^{-1} found in the supernatant of *B. pumilus* cultures (Degrassi *et al*., 1998). The enzyme was expressed and released due to cell lysis into LB medium and was purified from 1 l culture supernatant as shown in Table 2. SDS-PAGE analysis of the purified rAXE showed a single band of approximately 40 kDa (Fig. 2), whilst the molecular mass determined by gel filtration was 190 kDa, suggesting a homotetrameric or homopentameric structure of the enzyme, in accordance with the molecular mass of the native AXE from *B. pumilus*. The pI of the rAXE was determined by analytical IEF and found to be approximately 4·8. Activity staining confirmed the acetyl esterase activity of the band, but showed also another band of activity at pH 6·0, not corresponding with any sharp band focused in the Coomassie blue-stained gel (Fig. 2). The pH optimum of the purified rAXE was 8·5 and the temperature optimum was 45 °C. However, the range of pH and temperature where the activity was

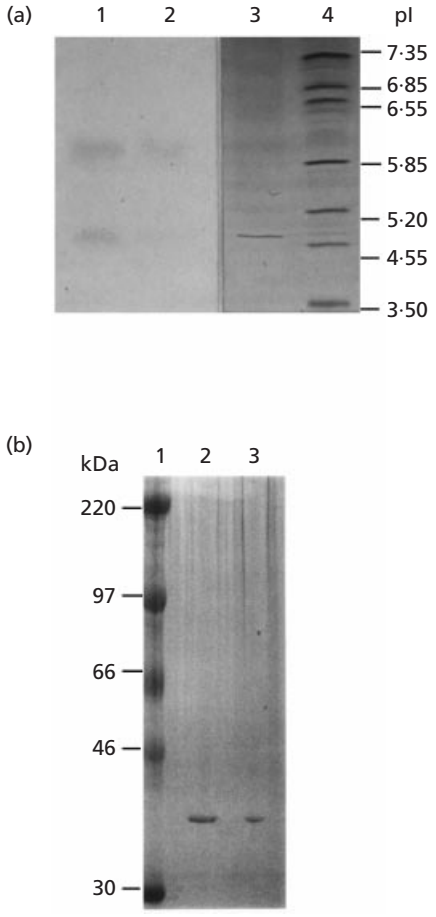

Fig. 2. Analytical IEF (a) and SDS-PAGE (b) of the purified rAXE. (a) Lanes: 1 and 2, activity staining of 10 μg (1) and 2 μg (2) of purified rAXE; 3, Coomassie blue staining of 10 μg purified rAXE; 4, pI markers, consisting of myoglobin (7·35), myoglobin (6·85), human carbonic anhydrase B (6·55), bovine carbonic anhydrase (5·85), β-lactoglobulin A (5·2), soybean trypsin inhibitor (4·55) and amyloglucosidase (3·5). (b) Lane 1, molecular mass standard, consisting of myosin (220 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (66), ovalbumin (46) and carbonic anhydrase (30); lane 2, 20 μg rAXE; lane 3, 5 μg rAXE.

Table 3. Substrate specificity and specific activity of rAXE

Apparent kinetic values are given for acetylated xylan, xylose and glucose.

Substrate	K_m (mM)	V_{max} (mmol min^{-1} mg^{-1})	V_{max}/K_m	Specific activity (U mg^{-1})
Acetylated xylan (*B. pumilus* AXE)				41 ± 8
Acetylated xylan (rAXE)				13 ± 5
Xylose tetraacetate	8·6 ± 3·5	550 ± 80	64	248 ± 27
Glucose pentaacetate	5·7 ± 2·5	327 ± 75	57	194 ± 18
α-Naphthyl acetate	1·2 ± 0·1	264 ± 47	220	144 ± 14
4-Methylumbelliferyl acetate	0·5 ± 0·2	98 ± 39	196	67 ± 17
p-Nitrophenyl acetate	0·5 ± 0·2	126 ± 36	252	88 ± 23
Cephalosporin C	2·1 ± 1·1	37 ± 11	18	26 ± 3
7-Aminocephalosporanic acid	5·3 ± 2·4	254 ± 82	48	179 ± 32

optimal was 7·5–9·0 and 42–65 °C, respectively, in accordance with the previously reported data for the AXE from *B. pumilus* (Degrassi *et al*., 1998). The kinetic parameters were determined using the purified rAXE on several acetylated substrates and compared with the same parameters previously reported (Degrassi *et al*., 1998), showing that the recombinant enzyme is at least as effective as the original one (Table 3). The best substrates for the rAXE were α-naphthyl acetate and *p*-nitrophenyl acetate, according to the V_{max}/K_m values, whilst the highest specific activity was on xylose tetraacetate (Table 3). It was also found that the specific activity of the recombinant AXE on α-naphthyl acetate (Table 3) was not lower than that of native AXE (Degrassi *et al*., 1998).

B. pumilus AXE has cephalosporin deacetylase activity

Due to the high level of identity between the *B. pumilus* AXE and the cephalosporin C deacetylase from *B. subtilis*, we tested the ability of rAXE to catalyse the deacetylation of cephalosporin C. We found that the recombinant enzyme can catalyse the reaction and that the specific activity is similar to the one reported for another AXE, from *Thermoanaerobacterium* sp., which shows amino acid sequence similarity to cephalosporin C deacetylase (Table 3). However, this value is lower than the one reported for the cephalosporin C deacetylase from *B. subtilis* (Mitsushima *et al*., 1995). The enzyme seems to be catalytically more specific for 7-aminocephalosporanic acid than for cephalosporin C, as suggested by the higher V_{max}/K_m, with also a higher specific activity (Table 3).

DISCUSSION

AXE is an accessory enzyme absolutely required for the complete mineralization of many xylans. In contrast to the considerable data available on xylanases and xylosidases, little information is available on AXEs; this report being to our knowledge only the second one describing a gene encoding a secreted bacterial AXE protein. In this study, we have continued our previous studies and report here the cloning and characterization of the *B. pumilus* acetyl xylan esterase gene (*axe*). In addition, we also report the heterologous expression, purification and characterization of the rAXE protein from *E. coli*.

The gene was isolated using oligonucleotides designed on the basis of the amino acid sequences obtained from the purified AXE from *B. pumilus* (Degrassi *et al*., 1998). The *axe* gene was identified in a 2·2 kb *Eco*RI fragment; it encodes a protein of 320 amino acids with a calculated molecular mass of 36 kDa. Interestingly, the protein showed no significant similarity with most identified AXEs; however, it displayed high identity, approximately 40 %, with two recently identified AXEs of *Thermoanaerobacterium* and *Thermotoga maritima* (Fig. 1). The highest identity (76 %) was observed with the cephalosporin C deacetylase of *B. subtilis*. This identity was not only in the primary structure; the rAXE had enzyme activity towards cephalosporin C and 7-aminocephalosporanic acid. Thus, the two proteins also have functional similarity. This enzyme activity was also observed for the rAXE of *Thermoanaerobacterium* (Lorenz & Wiegel, 1997). This family of AXEs have an esterase domain located at a similar position (Fig. 1). It was also observed that all four proteins are of similar size and display regions of identity throughout the whole protein. Interestingly, there is a stretch of 10 amino acids, PPSTVFAAYN, located very near the C terminus, which is identical in all four proteins (Fig. 1).

The AXE of *B. pumilus* was purified and characterized from supernatant (Degrassi *et al*., 1998); thus, the enzyme in its parent strain is secreted, as expected due to the function on acetylated xylan. Exported proteins are synthesized initially as preproteins with an amino-terminal extension; this signal peptide, which distinguishes the secreted proteins from cytoplasmic ones, is needed for targeting to the export pathway (Simonen & Palva, 1993). It is surprising that the *B. pumilus* AXE does not have an apparent signal sequence

as we have found that the parent strain secretes this enzyme. The AXE protein might possess an internal signal sequence. However, in prokaryotes, to our knowledge an internal signal sequence has not been reported; thus, further studies are needed to understand how this protein is secreted in *Bacillus* species. Interestingly, the *Thermoanaerobacterium* AXE protein and the cephalosporin C deacetylase of *B. subtilis* are reported not to be secreted (Lorenz & Wiegel, 1997), whereas the putative AXE of *Tht. maritima* has not been characterized.

The *axe* gene was successfully expressed in *E. coli* when cloned in pBluescript II SK(+); the expression was efficient, however, it led to a certain amount of cell lysis. The rAXE was not secreted in *E. coli* since the protein is of Gram-positive origin and thus cannot be secreted through the two membranes of Gram-negative bacteria. It was observed that the expression of the *axe* gene in pBluescript was not due to the *lac* promoter as the *axe* gene was cloned in the opposite orientation to this promoter; the expression was therefore due to its own promoter.

The characterization of the purified recombinant enzyme showed that it was highly similar to the AXE purified from *Bacillus pumilus* in terms of pH and temperature optima, and stability, molecular mass and pI. The heterologous rAXE protein was also assembled as a homotetramer or homopentamer of approximately 190 kDa, with a pI identical to the *B. pumilus* AXE. The N-terminal amino acid sequence of the rAXE was also found to be 100% identical with the deduced sequence from the gene. However, the IEF of the rAXE showed another band of activity at pI higher than expected not corresponding to any focused protein (Fig. 2), suggesting that the process of assembly and folding of the homopolymer in the heterologous system might be incomplete, perhaps due also to the high protein expression level.

Substrate specificity confirmed what was previously reported by Lorenz & Wiegel (1997), with the highest specific activity for rAXE found on xylose tetraacetate. We could detect activity on acetylated xylan but much lower than in *B. pumilus*. As observed in *Thermoanaerobacterium*, the expression of AXE in *E. coli* leads to the reduction or loss of activity on acetylated xylan. This might be explained by problems in folding of the homopolymeric protein in the heterologous system, as suggested also by the result of IEF, with a consequent reduced affinity or binding capacity for the complex substrate.

Further investigation therefore needs to be carried out to understand the specificity of this class of enzymes and their mechanism of regulation, in relation to the real metabolic function. In fact, the gene encoding the acetyl esterase in *B. pumilus*, which showed activity on acetylated xylan and was also found to be induced by this substrate, is more similar to the *B. subtilis* cephalosporin C deacetylase than to the *Thermoanaerobacterium* or *Thermotoga* AXEs. Furthermore, this gene has esterase activity on several substrates. These observations raise the question whether the primary function of the AXE of *B. pumilus* reported here is deacetylation of acetyl xylan. On the basis that the enzyme is secreted and that its production is induced by xylan and corn cob (Degrassi *et al*., 1998), we postulate that deacetylation of xylan is a primary function for this enzyme. This AXE belongs to esterase family 7 (Coutinho & Henrissat, 1999). The other three enzymes that belong to this class have similar characteristics; the AXE1 of *Thermoanaerobacterium* has broad substrate specificity, it is not clear whether this enzyme is secreted, and indications are that it is an internal enzyme having its primary activity on acetylated xylo-oligomers (Lorenz & Wiegel, 1997). The other characterized enzyme of this family is the cephalosporin C deacetylase of *B. subtilis*, which has been identified for its activity on cephalosporins; it is possible that its primary biological function is not known. In general, it appears that this class of enzymes has a broad substrate specificity and that possibly the enzymes have divergently evolved from a common ancestral gene. Further work is required to understand further the mode of action of this new family of esterases.

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