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Cloning, Sequences, and Characterization of Two Chitinase Genes from the Antarctic *Arthrobacter* sp. Strain TAD20: Isolation and Partial Characterization of the Enzymes

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Arthrobacter **sp. strain TAD20, a chitinolytic gram-positive organism, was isolated from the sea bottom along the Antarctic ice shell. *Arthrobacter* sp. strain TAD20 secretes two major chitinases, ChiA and ChiB (*Ar*ChiA and *Ar*ChiB), in response to chitin induction. A single chromosomal DNA fragment containing the genes coding for both chitinases was cloned in *Escherichia coli*. DNA sequencing analysis of this fragment revealed two contiguous open reading frames coding for the precursors of *Ar*ChiA (881 amino acids [aa]) and *Ar*ChiB (578 aa). *Ar*ChiA and *Ar*ChiB are modular enzymes consisting of a glycosyl-hydrolase family 18 catalytic domain as well as two and one chitin-binding domains, respectively. The catalytic domain of *Ar*ChiA exhibits 55% identity with a chitodextrinase from *Vibrio furnissii*. The *Ar*ChiB catalytic domain exhibits 33% identity with chitinase A of *Bacillus circulans*. The *Ar*ChiA chitin-binding domains are homologous to the chitin-binding domain of *Ar*ChiB. *Ar*ChiA and *Ar*ChiB were purified to homogeneity from the native *Arthrobacter* strain and partially characterized. Thermal unfolding of *Ar*ChiA, *Ar*ChiB, and chitinase A of *Serratia marcescens* was studied using differential scanning calorimetry. *Ar*ChiA and *Ar*ChiB, compared to their mesophilic counterpart, exhibited increased heat lability, similar to other cold-adapted enzymes.**

Chitin, the second most abundant biopolymer in nature next to cellulose, is an insoluble homopolysaccharide composed of β-1,4-linked *N*-acetylglucosamine (GlcNAc) residues. This polysaccharide is found in fungi, algae, and especially in the exoskeletons of insects and crustaceans. The turnover of chitin in the aquatic biosphere is enormous and mediated by chitinolytic bacteria (37). Chitinases (EC 3.2.1.14) hydrolyze the β-1,4-linkages in chitin, yielding predominantly *N-N′*-diacetyl chitobiose, which is further degraded by chitobiases to the GlcNAc monomer.

Several chitinases from bacteria have been cloned and expressed in *Escherichia coli* (6, 7, 18, 35). Furthermore, the structure of two chitinases has been elucidated (16, 23). Based on the amino acid sequence similarity of their catalytic domains, chitinases are classified into two unrelated families in the glycosylhydrolase classification system (15). Family 18 includes chitinases from bacteria, fungi, animals, and certain plants, while family 19 comprises chitinases of plant origin. Bacterial chitinases generally consist of multiple functional domains, such as chitin-binding domains (ChBDs) and fibronectin type III-like domains, linked to the catalytic domain. The importance of the ChBDs in the degradation of insoluble chitin has been demonstrated for several bacterial chitinases (4, 20, 29, 34).

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Psychrophilic microorganisms growing at ~5°C can be found in several permanently cold environments. Psychrophilic enzymes produced by such microorganisms display a high specific activity at low and moderate temperatures and are most often, if not always, associated with high thermosensitivity (14). These properties can be extremely useful for various applications. During the last years, several psychrophilic enzymes have been isolated (11, 13, 24, 25, 31), and the structure of four of them has been elucidated (1, 3, 19, 26).

In this study, we report cloning, sequence, and characterization of the genes coding for the precursors of two chitinases, *Ar*ChiA and *Ar*ChiB, from the Antarctic, aerobic gram-positive *Arthrobacter* sp. strain TAD20. The purification and partial characterization of the enzymes from the native strain are also described.

MATERIALS AND METHODS

Bacterial strains, plasmids, and enzymes. The chitinolytic strain TAD20 was isolated from sea sediments at the Dumont d'Urville Antarctic station (60°40′ S, 40°01′ E) in 1993. It was identified as an *Arthrobacter* sp. in the Laboratory of Microbiology (Jean Swings) at the University of Ghent (Belgium) by analysis of fatty acid composition and comparison of the profile with the MIDI database. Selection for chitinolytic activity was carried out on Marine agar 2216E (Difco) containing 1% colloidal chitin prepared as described by Hsu and Lockwood (17). *E. coli* X11-Blue was purchased from Stratagene. Reagents for bacterial media were from Difco. The pSP72 plasmid was purchased from Promega. The enzymes for molecular biology were obtained from Stratagene, Boehringer Mannheim, and Gibco-BRL and used according to the instructions of the manufacturers.

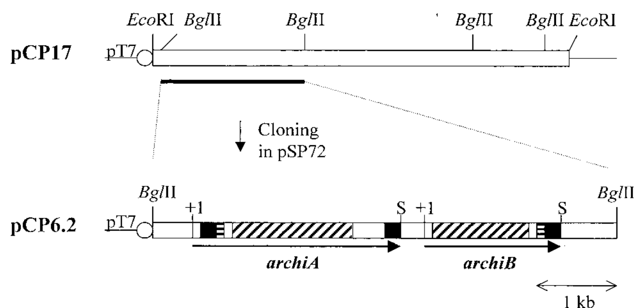

FIG. 1. Cloning of *archiA* and *archiB*. The black boxes in *archiA* and *archiB* correspond to ChBDs, the boxes with horizontal streaks correspond to Pro/Thr-rich regions, and the boxes with diagonal streaks correspond to the catalytic domains. The circles labeled pT7 show T7 promoter. S, stop codon.

Effect of temperature on growth of *Arthrobacter* sp. strain TAD20 and enzyme secretion. *Arthrobacter* sp. strain TAD20 was cultivated in nutrient broth (Difco) supplemented with 0.1% colloidal chitin in order to measure enzyme secretion at various temperatures. TAD20 was cultured aerobically at 4°C under vigorous shaking in 500-ml Erlenmeyer flasks containing 100 ml of medium. Growth was monitored by turbidity (optical density) measurements at 580 nm. Assays of chitinase were carried out using 0.1 mM *p*-nitrophenyl *N,N'*-diacetylchitobiose (pNP-chitobiose) (Sigma) using conditions described under the enzyme assay section. Activities are expressed as micromoles of substrate hydrolyzed per milliliter of sample.

Microorganism cultivation. The Antarctic strain was cultivated in shake cultures for 5 days at 5°C in 3 liters of medium consisting of 5 g of Bactotryptone, 1 g of yeast extract, 33 g of sea salts, and 1 g of colloidal chitin (pH 7.3) per liter (17) to induce secretion of the enzymes.

Enzyme purification. After centrifugation of the cell culture at 11,000 × *g* for 15 min, the supernatant was concentrated up to 400 ml and dialyzed against 20 mM Tris-HCl (pH 6.5) using a Minitan tangential flow ultrafiltration unit (Millipore) fitted with PTCGC membranes (10-kDa cutoff). The sample was then loaded on a Q_{FF}-Sepharose column (2 by 20 cm) equilibrated in the abovementioned buffer. The flowthrough was dialyzed against 20 mM Tris-HCl (pH 8), loaded on another Q_{FF}-Sepharose column (2 by 20 cm) equilibrated in 20 mM Tris-HCl (pH 8), and eluted with an NaCl linear gradient (0 to 200 mM, 250 to 250 ml). *Ar*ChiA active fractions were pooled and stored at 5°C. *Ar*ChiB active fractions in the flowthrough were concentrated to 10 ml, applied on a Sephacryl S-200 column (2.5 by 100 cm), and eluted with 20 mM Tris-HCl–100 mM NaCl (pH 7.5). Active fractions corresponding to pure chitinase B (± 82 mg) were pooled and stored at 5°C. Under these conditions, the enzymes were stable for at least 3 months.

Cloning and sequencing. All general techniques used were described by Sambrook et al. (27). Genomic DNA of *Arthrobacter* sp. strain TAD20 was digested with *Eco*RI, and the resulting fragments were ligated to *Eco*RI-cleaved and alkaline phosphatase-treated pSP72. The recombinant plasmids were used to transform *E. coli* XL1-Blue competent cells. Transformants were grown at 18°C on Luria-Bertani (LB) agar plates containing 100 μg of ampicillin/ml.

Mature colonies were transferred on nylon membranes (Amersham Life Science), set down on a paper (Wattman), and wetted with a solution of 0.01 mM 4-methylumbelliferyl *N,N'*-diacetylchitobiose (4-MU-chitobiose), 4-methylumbelliferyl *N,N',N'*-triacetylchitotriose (4-MU-chitotriose) (Sigma), and 20 mM HEPES (pH 7.5). Hydrolysis of these substrates results in the liberation of fluorescent 4-methylumbelliferone. Three identical positive clones carrying a 17-kb insert (termed pCP17) were identified by a fluorescent halo when visualized under UV light. A 6.2-kb *Bgl*II fragment subcloned from pCP17 was found to carry the DNA coding for *Ar*ChiA and *Ar*ChiB. It was ligated with pSP72 digested by *Bgl*II to give pCP6.2.

The nucleotide sequence of the 6.2-kb fragment was determined by a subcloning strategy and by gene walking with custom sequencing primers using the dideoxy chain termination method (28) on denatured double-stranded DNA templates with an ALF DNA sequencer (Pharmacia) and fluorescein-labeled primers.

Enzyme assay. Chitinolytic activity was routinely assayed at 25°C using 0.1 mM pNP-chitobiose (Sigma) as the substrate for *Ar*ChiA and *Serratia marcesens* chitinase A (*Sm*ChiA) and 0.1 mM pNP-chitotriose (*p*-nitrophenyl-*N,N',N''*-triacetylchitotriose) (Sigma) as the substrate for *Ar*ChiB in 20 mM HEPES (pH 7.5). Activities were recorded in a thermostated Uvicon 860 spectrophotometer (Kontron) and calculated on the basis of an extinction coefficient for *p*-nitrophenol of 14,700 $(M \times cm)^{-1}$ at 405 nm. For comparative studies, preliminary experiments were performed to determine the effect of pH, buffer composition, and monovalent and divalent ions on the activity and stability of *Ar*ChiA, *Ar*ChiB, and *Sm*ChiA. The thermal stability of the enzymes was measured by incubating the enzymes at 50°C at a concentration of 10 μg/μl in the corresponding optimal buffer for stability, i.e., 20 mM bis-Tris (pH 6.1) for *Ar*ChiA, 20 mM HEPES (pH 7.3) for *Ar*ChiB, and 20 mM HEPES (pH 6.6) for *Sm*ChiA. Aliquots were taken at different times, and enzyme activity was measured using the routine assay conditions described above.

Sequence analysis. Similarity searches were performed with updated versions of the Blast (2) and the Fasta (22) programs, using the facilities of the Greek

FIG. 2. Flanking sequences of *archiA* and *archiB*. The boxed sequences are inverted repeats that are potential sites for DNA-binding proteins. The Shine-Dalgarno sequence (SD) and the putative terminators are indicated.

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ArChiA  : EQRVIG-YFTSWRT-GENGAPRYLASDIPWKKLSHINYAFAHIDKNNKVSVG-AETPGNA   236
VfEndoI : PRRVIG-YFTSWRA-GDDDQTAYLVKDIPWEQLTHINYAFVSIGSDGKVNVGDVNDANNA   376
ArChiB  : GYRNVG-YFAQWGVYGRAFQAKQLDVSGTAKNLTHINYSFGNI---------------NN    98
BcChiA  : SYKIVG-YYPSWAAYGRNYNVADIDPT----KVTHINYAFADIC-WNGIHGNPDPSGPNP    96
SmChiA  : SGKVVGSYFVEWGVYGRNFTVDKI----PAQNLTHLLYGFIPICGGNGINDSLKEIEGS-   210
                                                 F

ArChiA  : ATDLTW----------PGGCGAEMDPTL---------------------PYKGHLNQLKK   265
VfEndoI : AVGKEW----------D---GVEIDPTL---------------------GFKGHFGALAT   402
ArChiB  : QTLTCFMANKAQGTG-PNGSDGAGDAWADFGMGYAADKSVSGKADTWEYPLAGSFNQLKQ   157
BcChiA  : VTWTC-QNEKSQTINVPNGTIVLGDPWIDTGKTFA--------GDTWDQPIAGNINQLNK   147
SmChiA  : -----FQALQRSCQGREDFKVSIHDP-------FAALQKAQKGVTAWDDPYKGNFGQLMA   258

ArChiA  : FKKENPGVKTLVSVGGWAETGGHFAADGTRVGDGGFYTLTE------SQAKIDTFSDSAV   319
VfEndoI : YKQKY-GVKTLISIGGWAETGGHFDNDGNRVADGGFYTMTTNADGSINQQGIETFADSAV   461
ArChiB  : LKAKNPKLKVMISLGGWTWSKNFSKAAATEQSRQKL---VSSCIDLYIKGNLPNFEGRGG   214
BcChiA  : LKQTNPNLKTIISVGGWTWSNRFSDVAATAATREVF---ANSAVDFLRKYN---------   195
SmChiA  : LKQAHPDLKILPSIGGWTLSDPFF-FMGDKVKRDRF---VGSVKEFLQTWK---------   305
                      S GG

ArChiA  : EFIRKYGFSGVDIDYEYATSNSKAGNPDDFAISEPRRAVLFENYMKLMKTLREKLDKASV   379
VfEndoI : EMMRKYRFDGLDIDLRISNIDGGTGNPDDTAFSESRRAYLMNSYHELMRVLREKLDVASA   521
ArChiB  : AGAAAGIFDGIDIDWEWPGTNSGLEGNGVDTFNDR------ANFKALLAEFRKQLDAYGS   268
BcChiA  : -------FDGVDLDWEYP-VSGGLDGNSKRP-EDK------QNYTLLLSKIREKLDAAGA   240
SmChiA  : ------FFDGVDIDWEFPG-GKGANPNLGSP-QDG------ETYVLLMKELRAMLDQLSA   351
                     D D E

ArChiA  : ADGTYYQLTVAAPASGWLLRGM---EAHQVVKYLDFVNMMSYDLHGAWN-NFVGGNPALF   435
VfEndoI : QDGVHYMLTIAAPSSAYLLRGM---ETMAVTQYLDYVNIMSYDLHGAWN-DHVGHNAALY   577
ArChiB  : TNNKKYVLSAFLPANPADIDAGGWDDPANFKSL-DFGSIQGYDLHGAWNPTLTGHQANLY   327
BcChiA  : VDGKKYLLTI---ASGASATYAANTELAKIAAIVDWINIMTYDFNGAWQ-KISAHNAPLN   296
SmChiA  : ETGRKYELTSAISAGKDKIDKVAYNVAQN---SMDHIFLMSYDFYGPFDLKNLGHQTALN   408
                                            D    M  D  G

ArChiA  : DDGKDPELAAGGVYNANK--GIGYLNSDWAYHYFRGAMPAGRINLGVPFYTRGWTNVQGG   493
VfEndoI : DTGKDSELAQWNVYGTAQYGGIGYLNTDWAFHYFRGSMPAGRINIGVPYYTRGWQGVTGG   637
ArChiB  : D---DPADPR---EPSKKFSADKAVKK-----YLAAGIDPKQLGLGLAAYGRGWTGAKNV   376
BcChiA  : Y---DPAASAAGVPDANTFNVAAGAQG-----HLDAGVPAAKLVLGVPFYGRGWDGCAQA   348
SmChiA  : ---------APAWKPDTAYTTVNGVNA-----LLAQGVKPGKVVVGTAMYGRGWTGVNGY   454

ArChiA  : TNGLYGSSVLADQNKCAPGTGPKAGGNSKCGDGAGGIDNLWHDSDPMGGELAAGANPIWH   553
VfEndoI : DNGLWGAR-LAKSKRVSNRYG--EGEKNNCGYGATGLDNMWHDVNAAGDEMGAGSNPMWH   694
ArChiB  : --S----------------------PWGPATDGAP-------------------------   387
BcChiA  : GNG----------------------QYQTCTGGSS-------------------------   361
SmChiA  : QNNI---------------------PFTGTATGPV-------------------------   468

ArChiA  : VLNLQKGVVGDYAASYG-SPTGA-LKGTYTRHFDNVTKNEWWWNDTTKTFLSGDADQAIQ   611
VfEndoI : AKNLEHGIWGSYLAVYGLDPTTAPLVGTYARNYDSVAIAPWLWNAEKKVFLSTEDKQSID   754
ArChiB  : ------GTYEKANEDYDKL------KTLGTDHYDAATGSAWRYDGT--QWWSYDNIATTK   433
BcChiA  : -----VGTWEAGSFDFYDLEANYINKNGYTRYWNDTAKVPYLYNASNKRFISYDDAESVG   416
SmChiA  : -----KGTWKNGIVDYRQIAGQFMSGE-WQYTYDATAEAPYVFKPSTGDLITFDDARSVQ   522

ArChiA  : AKADYVADKGLGGVMIWELAGDYE-Y------------------NASKGQYEMGSTLVNK   652
VfEndoI : VKADYVIDKEIGGIMFWELAGDYNCYVLDANGQRTSIDSTEQACESGQGEYHMGNTMTKA   814
ArChiB  : QKTDYIVSKGLGGGMWWELSGDRNGELVGAM-SDKFRAAAPGPVTEAAP--PSTTNPTPT   490
BcChiA  : YKTAYIKSKGLGGAMFWELSGDRNKTLQNKLKADLPTGGTVPPVDTTAPSVPGNARSTGV   476
SmChiA  : AKGKYVLDKQLGGLFSWEIDAD-NGDILNSMNASLGNSAGVQ*                    563
                          W    D
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FIG. 3. Sequence alignment of *Ar*ChiA, *Ar*ChiB, several bacterial chitinases, and a chitodextrinase. The alignment was constructed using the multiple alignment program (PILEUP) from the Greek EMBnet Node. *Vf*EndoI, chitodextrinase of *V. furnissii*; *Bc*Chia, chitinase A of *B. circulans*; *Sm*ChiA, chitinase A of *S. marcescens*. Amino acid numbering is indicated on the right. The amino acids under the sequence alignment are the consensus sequence determined from the alignment of five bacterial chitinases as described in the text. The catalytic residue is double underlined.

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ChBDA1    :       SPSGTTTTKACA-TVAPASSYSSNAVVSHQSVNYSAKWWTQNNVPGSDPWGPWTSQGTCGTVTDPTPTP 102
ChBDA2    : GTGGGNGAGPVESCNG-AVTWSAAAVYTGGAVVAHAGSSYKAKWWTQGEVPGSGAANAWGSPILCGK*       881
ChBDB     : TVTPTTPPTTPPAGSGCAAPWSATATYLGGSKVSHGGISYKAKWWTLNEKPGASQWGPWEATGACN*       881
ChBDaltso : VNAVDTTFNVTIKDGAEYPTWDRSTVYVGGDRVIHNSNVFEAKWWTQGEEPGTADV--WKAVTN*         820

                                W   T Y  G  VS   G  @ A W@T G  P T       W
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FIG. 4. Sequence alignment of the ChBDs of *Ar*ChiA (ChBDA1 and ChBDA2) and *Ar*ChiB (ChBDB) and of *Alteromonas* sp. strain O-7 chitinase A (ChBDaltso). The alignment was constructed using the multiple alignment program (PILEUP) from the Greek EMBnet Node. Amino acid numbering is indicated on the right. The amino acids under the sequence alignment are the consensus sequence determined from the alignment of putative bacterial ChBDs as described in the text. @, aromatic residue.

EMBnet Node. The Genedoc program (http://www.psc.edu/biome/genedoc) was used for editing and shadowing the sequence alignment.

Other methods. Protein concentration was measured using the bicinchoninic acid protein assay reagent (Pierce). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was run as described by the supplier of the electrophoresis equipment (Hoeffer Scientific Instruments). Isoelectric focusing was run on a Fast System Separation and Control Unit from Pharmacia using gels with a 3-10 pH gradient. The NH_2-terminal amino acid sequence of *Ar*ChiA and *Ar*ChiB was determined using a pulsed liquid-phase protein sequenator (Applied Biosystems 477A) equipped with an on-line 120A phenylthiohydantoin analyzer. C-terminal amino acid sequences were obtained on a Procise 494CT sequencer (Applied Biosystems, Perkin-Elmer Division) equipped for alkylthiohydantion analysis.

Differential scanning calorimetry (DSC) measurements were performed using a MicroCal MCS-DSC instrument at a scan rate of 60 K h^{-1} and under a nitrogen pressure of 2 atm. Samples were dialyzed overnight against the appropriate buffer, the dialysate being used in the reference cell and for buffer base line determination. Protein concentration after dialysis was ~4 mg/ml for *Ar*ChiA and *Ar*ChiB and ~2 mg/ml for *Sm*ChiA. Buffers used were those ensuring optimal stability for the enzymes, as previously described.

Denaturation curves for *Ar*ChiA and *Ar*ChiB were analyzed using MicroCal Origin software (version 2.9).

Nucleotide sequence accession numbers. The nucleotide sequences of *Ar*chiA and *Ar*chiB have been deposited and assigned accession numbers AJ250585 and AJ250586, respectively, in the EMBL database.

RESULTS

Effect of temperature on enzyme secretion. *Arthrobacter* strain TAD20 was grown at 4, 17, and 24°C. Chitinase was assayed as described in Materials and Methods. Highest activity was observed in supernatants of cultures incubated at 4°C, similar to other enzymes from psychrophilic bacteria (10). Chitinase activity could not be detected when TAD20 was grown at 17 or 24°C.

Cloning and sequencing of *archiA* and *archiB*. The structural genes for *Ar*ChiA and *Ar*ChiB were cloned from a genomic library of the Antarctic bacterium *Arthrobacter* sp. strain TAD20. In order to prevent thermal denaturation of the cloned product, *E. coli* transformants were grown at 18°C. Three thousand transformants were transferred on nylon membranes and screened for activity on a mixture of 4-MU-chitobiose and 4-MU-chitotriose. Three identical clones carrying a 17-kb insert (pCP17) were detected by the appearance of a fluorescent halo when exposed to UV light.

From subcloning, a plasmid containing a 6.2-kb fragment (pCP6.2) and conferring chitinase activity on both 4-MU-chitobiose and 4-MU-chitotriose substrates to transformed *E. coli* cells was isolated (Fig. 1) and sequenced on both strands. The nucleotide sequence revealed two open reading frames of 2,640 and 1,731 bp, coding for *Ar*ChiA (*archiA*) and *Ar*ChiB (*archiB*), respectively. Upstream of the ATG codon of those genes, a short sequence has been identified that may function as a Shine-Dalgarno ribosome-binding site; typical transcription initiation sequences were not identified (Fig. 2). Downstream of the stop codons of *archiA* and *archiB* are short inverted repeats which are putative terminators. Upstream of *archiA* and *archiB* are inverted repeat sequences which are potential sites for protein binding (21).

***Ar*ChiA and *Ar*ChiB peptide sequence analysis.** The NH_2-terminal amino acid sequences of the purified *Ar*ChiA and *Ar*ChiB (ASPSGT and AAPPNTA respectively) allowed us to locate the signal peptidase cleavage site, which fulfills the −3, −1 rule of von Heijne (33); the leader peptides of *Ar*ChiA and *Ar*ChiB are composed of 34 and 38 amino acid residues, respectively. It also adopts the general pattern of prokaryotic signal sequences, i.e., a positively charged amino terminus followed by a hydrophobic core and a string of polar residues (36). Furthermore, C-terminal amino acid sequence analysis of *Ar*ChiA and *Ar*ChiB (ILCGK and TGACN, respectively) confirmed the C termini deduced from DNA translation. The deduced primary structures of the mature *Ar*ChiA and *Ar*ChiB consist of 846 and 539 amino acids with a predicted M_r of 89,415 and 57,123, respectively.

Blast analysis of the amino acid sequence of *Ar*ChiA and *Ar*ChiB revealed that both enzymes exhibit a catalytic domain as well as two and one ChBDs, respectively (Fig. 1). The catalytic domains of *Ar*ChiA (residues 183 to 653) and *Ar*ChiB (residues 57 to 473) exhibit 55 and 33% identity, respectively, with homologous regions of the chitodextrinase of *Vibrio furnissii* (18) and the chitinase A1 of *Bacillus circulans* WL-12, respectively (35) (Fig. 3). The identity of the catalytic domains

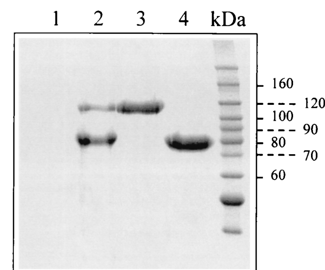

FIG. 5. SDS-PAGE of *Ar*ChiA and *Ar*ChiB secreted by *Arthrobacter* sp. strain TAD20. Lanes 1 and 2, 20 μl of the supernatants of cultures grown in the absence of chitin (lane 1) or in the presence of 0.1% colloidal chitin (lane 2). All cultures (10 ml) were grown at 5°C under identical conditions in a medium containing 2 g of yeast extract, 5 g of tryptone, and 33 g of sea salts per liter (pH 7.2). Colloidal chitin was prepared as described by Hsu and Lockwood (17). Lanes 3 and 4, purified *Ar*ChiA and *Ar*ChiB, respectively. The gel was stained using Coomassie brilliant blue.

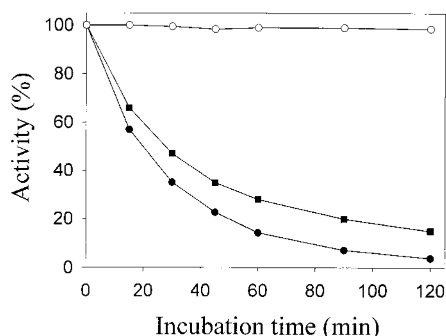

FIG. 6. Thermal stability of the chitinases of *Arthrobacter* sp. strain TAD20 and the mesophilic chitinase A of *S. marcescens*. *Ar*ChiA (●), *Ar*ChiB (■), and *Sm*ChiA (○) were incubated at 50°C for the indicated periods of time in 20 mM bis-Tris (pH 6.1) for *Ar*ChiA, 20 mM HEPES (pH 7.3) for *Ar*ChiB, and 20 mM HEPES (pH 6.6) for *Sm*ChiA.

of *Ar*ChiA and *Ar*ChiB with that of the crystallised chitinase A of *S. marcencens* (*Sm*ChiA) (23) is 29% in a 458-amino-acid (aa) overlap for *Ar*ChiA and 26.5% in a 475-aa overlap for *Ar*ChiB (Fig. 3).

At the N and C termini of *Ar*ChiA and at the C terminus of *Ar*ChiB, similar (ChBDs) occur, namely, ChBDA1 (residues 44 to 93) and ChBDA2 (residues 827 to 878) for *Ar*ChiA and ChBDB (residues 425 to 578) for *Ar*ChiB (Fig. 4).

Characterization of *Ar*ChiA and *Ar*ChiB. The apparent molecular masses on SDS-PAGE of the purified *Ar*ChiA (±110,000 Da) and *Ar*ChiB (±80,000 Da) (Fig. 5) were higher than those the estimated one from the DNA sequence translation (89,415 and 57,123 Da, respectively). The isoelectric points of *Ar*ChiA and *Ar*ChiB are 5.7 and 8.1, respectively. No ion was found to increase the activity of these enzymes, and they retain 100% of their activity in the presence of a 10 mM EDTA solution. Optimal buffer for activity was 20 mM HEPES (pH 7.3 to 8) for *Ar*ChiA and *Ar*ChiB. No ion was found to increase the stability of the enzymes from *Arthrobacter* or *S. marcescens*. Optimal buffer for stability was 20 mM bis-Tris (pH 6.1) for *Ar*ChiA and 20 mM HEPES (pH 7.3) for *Ar*ChiB.

Thermal stability. The denaturation curves of the psychrophilic and mesophilic enzymes were recorded at 50°C in the corresponding optimal buffers for stability (Fig. 6), showing that the psychrophilic chitinases are less stable than their mesophilic counterpart, a common characteristic of cold-adapted enzymes.

DSC. The denaturation curves of *Ar*ChiA, *Ar*ChiB, and *Sm*ChiA show single peaks with apparent T_ms of 54.3, 54, and 64.2°C, respectively (Fig. 7). Calculation of the areas under the heat absorption peaks determined the calorimetric denaturation enthalpy (ΔH_{cal}) of *Ar*chiB (415 kcal/mol), *Ar*ChiB (270 kcal/mol), and *Sm*ChiA (449 kcal/mol).

DISCUSSION

In this report, we describe for the first time the cloning, sequencing, and characterization of two chitinase genes from the Antarctic marine strain *Arthrobacter* sp. strain TAD20. This strain secretes mainly two chitinases, *Ar*ChiA (~10 mg/liter) and *Ar*ChiB (~40 mg/liter) in response to chitin induction (Fig. 5). A single 17-kb chromosomal DNA fragment of *Arthrobacter* sp. strain TAD20 containing the genes coding for the precursors of *Ar*ChiA and *Ar*ChiB was cloned in *E. coli* using a mixture of 4-MU-chitobiose and 4-MU-chitotriose in order to detect positive clones which appeared fluorescent under UV light. Attempts to screen the transformed cells on plates containing colloidal chitin (32) were unsuccessful, possibly due either to the fact that the cloned chitinases are not secreted by *E. coli* or that their expression level is under the detection limit (12).

Upstream of *archiA* and *archiB*, inverted repeat sequences were identified (Fig. 2). The marked differences between these sequences provide a good indication that *archiA* and *archiB* are regulated independently and that the mode of their regulation is different.

The *archiA* and *archiB* genes encode the precursors of modular chitinases composed of an N-terminal signal peptide, a catalytic domain of the glycosyl-hydrolase family 18, as well as two and one ChBDs, respectively (Fig. 1).

Chitinases follow the general acid-base catalytic mechanism (8), and from sequence comparison with *Sm*ChiA, Glu-335 of *Ar*ChiA and Glu-230 of *Ar*ChiB are predicted to be the catalytic residues, acting as proton donors (23, 30).

The N-terminal signal sequences of *Ar*ChiA (34 aa) and *Ar*ChiB (38 aa) are relatively long, a common characteristic of enzymes secreted by gram-positive organisms (35). The catalytic domain of *Ar*ChiA exhibits the best homology (55% identity) with a chitodextrinase of *V. furnissii*. However, *Ar*ChiA, in contrast to chitodextrinase, is active on insoluble chitin (18). Furthermore, *Ar*ChiA carries two ChBDs, while the chitodextrinase carries none, which is in support of the observed difference in substrate specificity (34). The catalytic domain of *Ar*ChiB exhibits the best homology (33% identity) with chitinase A of *B. circulans* and a low homology (26% identity) with *Ar*ChiA (Fig. 3).

The ChBDs of *Ar*ChiA and *Ar*ChiB are 50 to 60 residues long, similar to other ChBDs, and exhibit good homology (50% identity) with the ChBD of chitinase A from *Alteromonas* sp. (Fig. 4) (32). Sequence alignment of this ChBD with ChBDA1, ChBDA2, and ChBDB revealed a consensus sequence which appears to be quite well conserved in several bacterial ChBDs

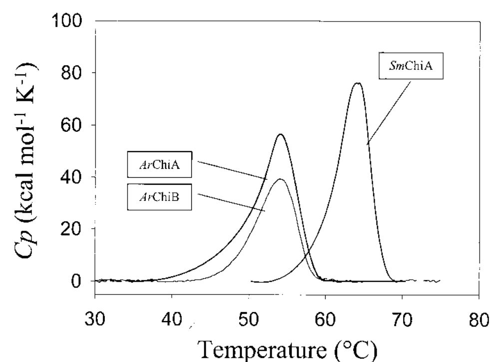

FIG. 7. Thermal unfolding of the chitinases of *Arthrobacter* sp. strain TAD20 and the mesophilic chitinase A of *S. marcescens*. Microcalorimetric records of *Ar*ChiA, *Ar*ChiB, and *Sm*ChiA. Experiments were performed in 20 mM bis-Tris (pH 6.1) for *Ar*ChiA, 20 mM HEPES (pH 7.3) for *Ar*ChiB, and 20 mM HEPES (pH 6.6) for *Sm*ChiA.

(4, 34). Furthermore, Trp and Tyr residues are conserved, suggesting that these aromatic side chains might be involved in the stacking against the pyranosyl rings of *N*-acetylglucosamine residues in chitin (5). Finally, ChBDA1 and ChBDB are linked to the catalytic domain via a long Pro/Thr-rich region, while ChBDA2 is linked via a 9-aa (815 to 823) sequence containing six glycines, both regions acting as flexible hinges.

The native *Ar*ChiA and *Ar*ChiB were purified to homogeneity employing conventional chromatographic techniques. The optimum pH was 7.3 to 8 for both enzymes. The apparent molecular masses of *Ar*ChiA and *Ar*ChiB as determined by SDS-PAGE were 110 and 80 kDa, respectively, higher than those estimated from the deduced amino acid sequence (Fig. 5). However, C-terminal amino acid analysis for both enzymes confirmed the C termini deduced from DNA translation.

Increased flexibility related to increased heat lability has been proposed to be the main structural feature of cold-adapted enzymes, allowing conformational changes necessary to reach the transition state enabling catalysis (14). The results obtained by thermal denaturation (Fig. 6) and DSC (Fig. 7) demonstrate that under optimal conditions, *Ar*ChiA and *Ar*ChiB are less stable than their mesophilic counterpart *Sm*ChiA. The psychrophilic enzymes exhibited remarkable thermal lability. Following incubation of *Ar*ChiA and *Ar*ChiB at 50°C for 60 min, the enzymes retained 18 and 30% of their original activity, respectively, while *Sm*ChiA retained almost 100% of the original activity. DSC denaturation curves show that, compared to *Sm*ChiA, the psychrophilic chitinases have a lower apparent T_m (54.3 and 54 for *Ar*ChiA and *Ar*ChiB, respectively, and 64.2 for *Sm*ChiA) as well as a significant lower calorimetric denaturation enthalpy per mole of residue. The nonsymmetrical shape of the denaturation curves is probably the result of a multitransition process combined with an aggregation phenomenon. For this reason, deconvolution of the denaturation curves into symmetrical components was not attempted.

The values of the denaturation enthalpy per residue are 490, 501, and 907 cal (mol of residue)$^{-1}$ for *Ar*ChiA, *Ar*ChiB, and *Sm*ChiA, respectively. Although these values are small, possibly due to aggregation phenomena, they are comparable to those found for the psychrophilic α-amylase from *Alteromonas haloplanctis* [525 cal (mol of residue)$^{-1}$] and the mesophilic α-amylase from *Bacillus amyloliquefaciens* [1,008 cal (mol of residue)$^{-1}$] (9).

The relationship between stability, specific activity, and flexibility for *Ar*ChiA and *Ar*ChiB is now under study in our laboratory.

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