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Molecular Cloning of an α-Glucosidase-like Gene from *Penicillium minioluteum* and Structure Prediction of Its Gene Product

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The *dexC* cDNA, which is expressed in dextran-containing medium by the filamentous fungus *Penicillium minioluteum*, was cloned and sequence characterized. The cDNA sequence comprises 1859 bp plus a poly (A) tail, coding for a predicted protein of 597 amino acids. The genomic counterpart was isolated by PCR, finding three introns in its sequence. The *dexC* gene was located by Southern blot in the same 9-kb fragment that the previously isolated dextranase-encoding gene (*dexA*). Sequence analysis revealed that the deduced DexC protein belongs to glycosyl hydrolase family 13, showing a high sequence identity (58%) with *Aspergillus parasiticus* α-1,6-glucosidase. In addition, the high sequence identity (51%) between DexC protein and oligo-1,6-glucosidase of *Bacillus cereus*, with three-dimensional (3D) structure determined, leads us to proposed a 3D model for the structural core of DexC protein.

Key Words: **glycosyl hydrolase; α-glucosidase; dextran; SequenceSpace analysis; *Penicillium minioluteum*.**

Dextran is an α-D glucopyranose homoglycan polymer, in which the main chain is formed by α-1,6 glucosidic linkages and may be branched via α-1,3. α-1,2 and α-1,4 glucosidic linkages are also found in dextran polymers. There are two major classes of enzymes which hydrolyze dextran polymers: [i] exodextranases (EC 3.2.1.70) that release either glucose or isomaltose from the non-reducing ends of dextran and [ii] endodextranases (EC 3.2.1.11) that hydrolyze the α-1,6 glucosidic linkages within the dextran polymer and between branch points to release smaller oligosaccharides (1). According to sequence similarities, exodextranases has been included into glycosyl hydrolase (GH) families 13, 27, and 49, whereas the endodextranases belong to GH families 66 and 49 (http://afmb.cnrs-mrs.fr/~pedro/CAZY/ghf.html).

The GH family 13 is poly-specific and comprises enzymes responsible of the hydrolysis of α-1,2; α-1,3; α-1,4 and α-1,6 glucosidic linkages. In these family are include α-glucosidases and oligo-α-1,6-glucosidases. α-glucosidase enzyme has been shown to preferentially hydrolize maltose (α-1,4 glucosidic linkages), whereas the oligo-α-1,6-glucosidases acts exclusively on α-1,6 linkage. α-glucosidases show also, transglycosylation activity. Different specificities in glucosidic linkage hydrolysis and α-glucosidases transglycosylation activity could be due to the differences in the structures of binding and catalytic sites of the enzymes (2).

The filamentous fungus *P. minioluteum* strain HI-4 synthesizes and secretes an endodextranase enzyme (α-1,6 glucan-6 glucanohydrolase, EC 3.2.1.11), which has been previously purified (3). The endodextranase-encoding cDNA was isolated by differential hybridization (4), and Northern blot experiments showed that its expression is regulated at the transcriptional level (5). Additional cDNA clones, expressed in dextran-induced cultures were also identified in the same differential hybridization experiment (4). Southern hybridization and restriction mapping analysis of selected clones revealed non-homologous cDNAs, corresponding to three different genes. Here, we describe the characterization of one of this cDNA clones and the isolation of its genomic counterpart, named *dexC*. The protein sequence, deduced from the nucleotide sequence, was compared with the protein sequences from non-redundant databases. The results of this comparison and the prediction of the 3D structure for the *dexC* gene product are also discussed. The proposed model will facilitate further studies concerning the structure of substrate-binding and catalytic sites of the enzymes into GH family 13.

The nucleotide sequence of the *dexC* gene encoding for a putative α-glucosidase from *Penicillium minioluteum* (HI-4) has been assigned Accession No. AJ278706 in the EMBL Nucleotide Sequence Database.

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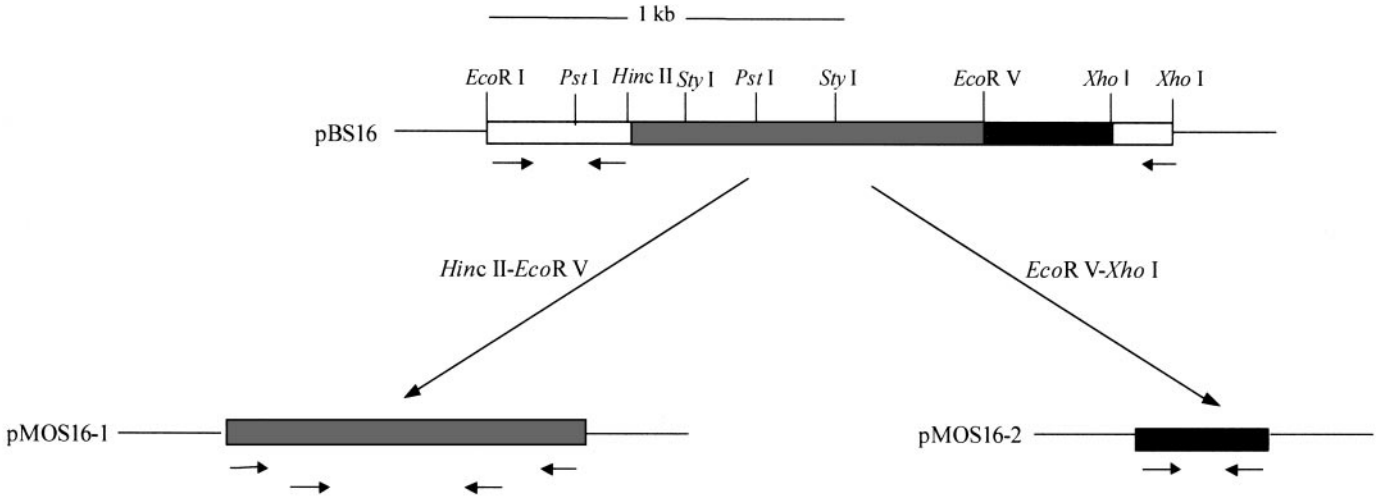

FIG. 1. Strategy followed to sequence the *dexC* gene. Arrows indicate the oligonucleotide primers used for sequence.

MATERIALS AND METHODS

Microbial strains and vectors. *P. minioluteum* HI-4 (6), a dextran-hydrolyzing fungal strain was used throughout this study. *Escherichia coli* strain TOP10 was used for the cloning and propagation of the genetic constructions. The plasmid pBS16, carrying the *dexC* cDNA, was isolated previously (4). The dextranase gene (*dexA*), used as probe in Southern blot experiments, was extracted from pBS25 plasmid (4). pMOS*blue* plasmid (Amersham, UK) was used as cloning vector of the PCR products and sequenced fragments.

Recombinant DNA techniques. Cloning, DNA manipulations, and *E. coli* transformation were done by standard techniques (7). High specific-activity labeling of hybridization probes was carried out by random hexamer priming (8) using [α-^{32}P]dATP (>3000 Ci/mmol, Amersham, UK). For Southern blot analysis, DNA was transferred to Hybond-N membranes (Amersham, UK), and treated as in Sambrook *et al.* (7). DNA sequence analysis was performed by dideoxy-chain termination method (9).

Isolation of P. minioluteum dexC gene. For the isolation of chromosomal DNA, *P. minioluteum* fungus was grown in liquid YPD medium (1% yeast extract, 2% bacto-peptone and 2% dextrose) at 28°C for two days. The mycelia were harvested, washed with water, and the genomic DNA was prepared as described by Raeder and Broda (10). The *P. minioluteum dexC* gene was amplified by PCR, using 100 ng of chromosomal DNA and 50 pmoles of oligonucleotide primers DexC1 (5′-AGATCTATCCTGCATCTTTCAAA-3′) and DexC2 (5′-ACGACTCTTCCATGTCTTCAAG-3′). After 30 cycles of amplification (denaturation: 94°C, 60 s; annealing: 55°C, 60 s; elongation: 72°C, 120 s), the amplified product was isolated from agarose gel and subcloned into pMOS*Blue* (Amersham,UK) to give plasmid pMDEXC.

Sequence analysis and structure prediction. cDNA nucleotide sequence was translated into protein sequence using the GeneRunner program (Hastings Software Inc., version 3.02). The deduced amino acid sequence was compared against SWISSPROT, PIR, PDB, and the CDS translations from GenBank, EMBL, DDBJ databases using the WWW BLAST service (http://www.ncbi.nlm.nih.gov:80/BLAST/). Multiple sequence alignments were done by CLUSTALW program (11). The 3D model, for the structural core of DexC, was created by WHAT IF program (12). To analyze the volume, surface area, and to identify those residues delineating the substrate-binding cavity, we used the WWW CAST service (http://sunrise.cbs.umn.edu/cast). The SequenceSpace method (13) was used to predict residues likely to be responsible for functional differences between protein subfamilies.

RESULTS AND DISCUSSION

The *dexA* cDNA encoding dextranase enzyme was isolated from a *P. minioluteum* cDNA library. This gene was expressed in dextran-containing medium but not when glucose was used as carbon source (4). Others cDNA clones were also specifically expressed in dextran containing medium, but not in glucose. Northern hybridization confirmed that these cDNA clones correspond to genes differentially expressed at different levels in these two culture conditions, and were represented in the cDNA library at different levels too (4). The most abundant corresponds to the dextranase-encoding gene (*dexA*) (4), the second one (*dexB*) shows sequence homology with sugar transporter proteins (manuscript in preparation), the third (*dexC*) is the aim of the present study, and the last one (*dexD*) revealed sequence homology with α-amylase proteins (manuscript in preparation).

Isolation and Characterization of the dexC Gene

For determination of the *dexC* sequence, we subcloned the 1-kb *Hinc*II-*Eco*RV and the 500-bp *Eco*RV-*Xho*I fragments of pBS16 (4) into the pMOS*Blue* vector and the resulting plasmids were called pMOS16-1 and pMOS16-2, respectively (Fig. 1). Inserts in plasmids pBS16, pMOS16-1, and pMOS16-2 were sequenced using a set of universal and specific oligonucleotide primers. To the *dexC* gene was assigned EMBL database Accession No. AJ278706.

The cDNA nucleotide sequence with the predicted amino acid sequence is shown in Fig. 2 and comprises 1859 bp plus a poly (A) tail. The open reading frame starting from the first ATG codon predicts a polypeptide of 597 amino acids.

To isolate *P. minioluteum dexC* gene we designed two oligonucleotides, DexC1 and DexC2, from the *dexC*

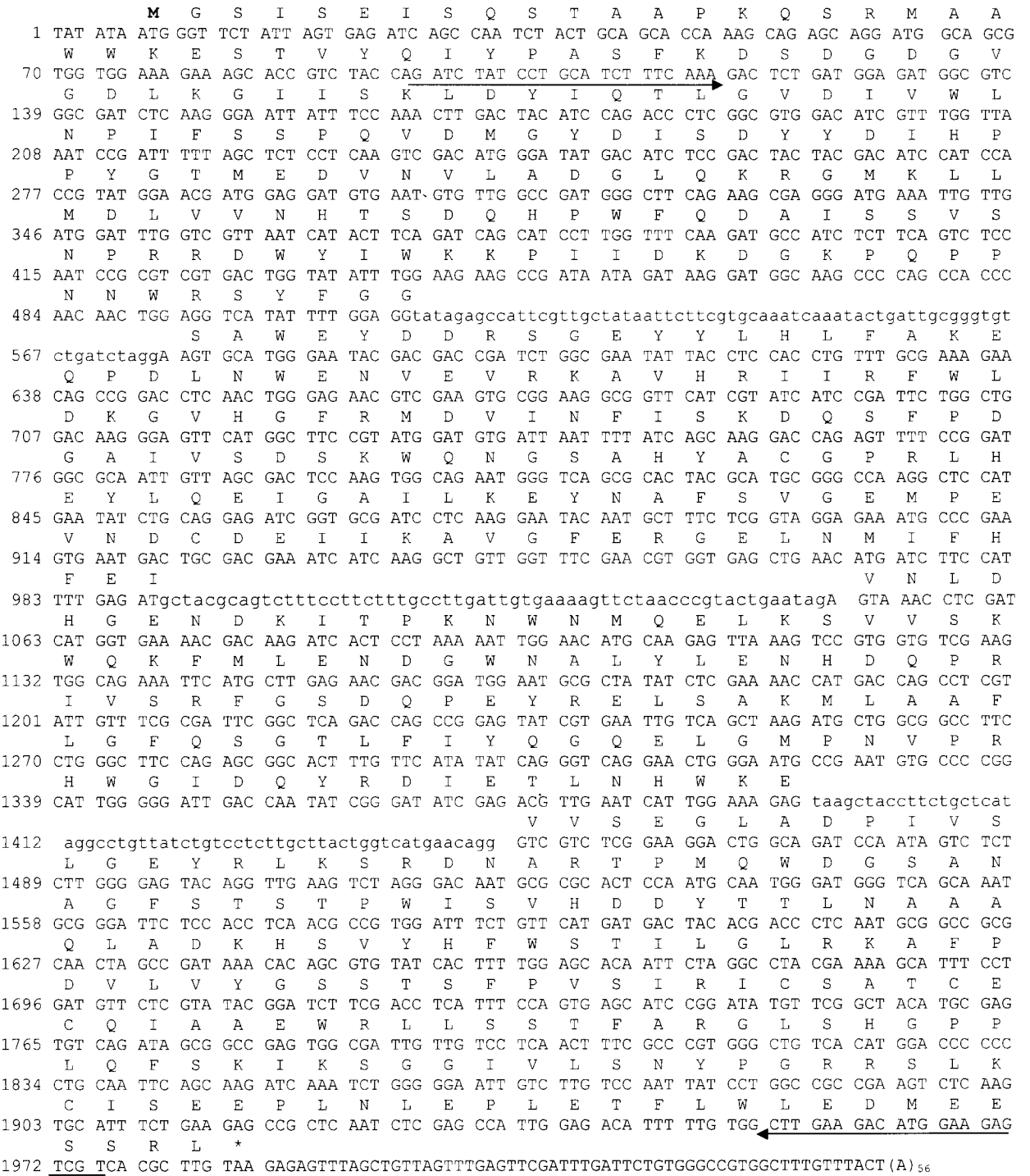

FIG. 2. Sequence of *dexC* gene from *P. minioluteum*. The oligonucleotides used for the isolation of *dexC* chromosomal gene are underlined. The introns are shown in lowercase letters. Predicted amino acid sequences from the first ATG are given using the one letter code.

cDNA sequence (sequences underlined in Fig. 2). PCR amplification of the 2-kb fragment was performed using *P. minioluteum* chromosomal DNA as template. The amplified DNA was cloned into pMOS*Blue* to give plasmid pMDEXC, which was used for sequencing, using the same strategy showed in Fig. 1.

Comparison of the cDNA and genomic sequences showed three introns of 67, 59, and 60 bp (lowercase letters in Fig. 2), respectively, seemingly distributed at random throughout the gene.

In order to map this gene in *P. minioluteum* genome, the chromosomal DNA was digested with different re-

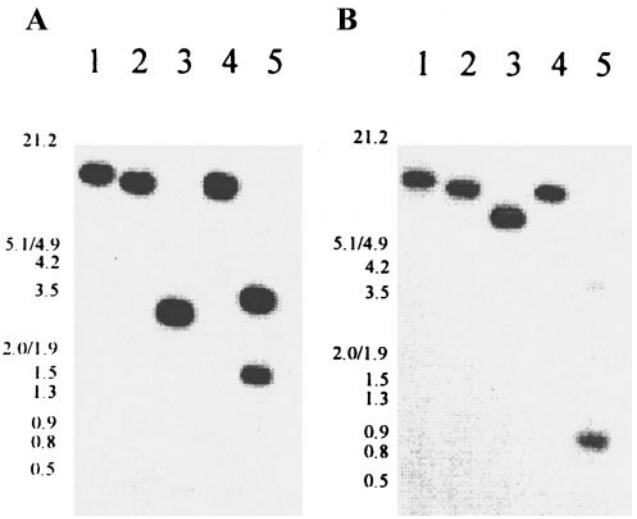

FIG. 3. Southern blot analysis of pUDEX plasmid using the *dexA* (A) and *dexC* (B) gene probes. The plasmid was digested with different restriction enzymes: lane 1, *Bam*HI; lane 2, *Eco*RI; lane 3, *Xba*I; lane 4, *Bam*HI- *Eco*RI; and lane 5, *Eco*RV.

striction endonucleases and analyzed by Southern blot, using the *dexC* and *dexA* cDNAs as hybridization probes.

When chromosomal DNA was digested with *Bam*HI or *Eco*RI restriction enzymes, both probes hybridize to band of 9 and 7 kb, respectively (data not shown). These results may indicate that both genes are present in the same DNA fragment. Since for the isolation of the *dexA* gene a 9-kb *Bam*HI fragment was cloned in the pUC19 plasmid (to give the pUDEX plasmid) (14) we also determined if this fragment contained both genes. pUDEX plasmid was digested with several restriction enzymes and probed against *dexA* and *dexC* genes, respectively. As shown in Fig. 3, both probes hybridized with the insert carried by the pUDEX and the hybridization patterns were the same as those obtained when chromosomal DNA was used. This result suggested that *dexA* and *dexC* genes are completely contained within the 9 kb *Bam*HI fragment. This was also corroborated when a PCR reaction was carried out using the oligonucleotides DexC1 and DexC2 and pUDEX plasmid as template.

Moreover, the cDNAs corresponding to *dexB* and *dexD* genes (homologous to sugar transport and α-amylase proteins, respectively) were used as probes in this experiment, showing that only the gene *dexB* hybridized with this fragment (data not shown). Taking into account that with the exception of the *dexD* gene, the other three genes are grouped in the same 9-kb *Bam*HI fragment, it is tentative to speculate that in *P. minioluteum* the genes necessary for dextran assimilation and hydrolysis are clustered. In filamentous fungi, gene clusters for the assimilation of alternative carbon sources as ethanol (15) and proline (16, 17) has been described. In *Saccharomyces cerevisiae* *MAL* genes are organized in a gene cluster (18), which comprises at least three of the genes involving in maltose assimilation. However, to our knowledge, there is no information available of gene clusters grouping glycosyl hydrolases in filamentous fungi, being this, the first report.

Sequence Analysis of the DexC Predicted Amino Acid Sequence

The comparison of the DexC deduced amino acid sequence with PIR, SWISS-PROT, and the CDS translations from GeneBank, EMBL and DDBJ databases, revealed a high sequence identity (43–58%) to α-1,6-glucosidases enzymes. Sequence similarities were also found with E-values better than e-100 for trehalose-6-phosphate hydrolase, α-phosphotrehalase treA, exo-α-1,4-glucosidase (maltase) and glucan 1,6-α-glucosidase (dextran glucosidase) (Table 1). All these homologous sequences belong to GH family 13 (19–21).

The GH families are established taking into account the amino acid sequence similarities, what supposes that enzymes belonging to the same family share a similar 3D structure and catalytic mechanism (19–21).

Figure 4A shows an alignment of the most conserved regions in oligo-1,6-glucosidases, dextran glucosidases and neopullulanases in comparison with DexC protein. All these enzymes hydrolyze α-1,6 glucosidic linkages. As result of the alignment were found several conserved regions, which correspond to some of the conserved domains (I, II, III, IV, V, VI and VII) found in the enzymes of GH family 13. This family is composed by more than 400 proteins (http://afmb.cnrs-mrs.fr/~pedro/CAZY/ghf_13.html), most of which are α-amylases. Several beta strands β2, β3, β4, β5, β7, and β8 of the α-amylase $(\beta/\alpha)_8$-barrel structure are found within the conserved regions. The Taka-amylase A amino acids D206, E230 and D297 (bold letters in Fig. 4A) located in beta strands β4, β5, and β7, respectively, are the catalytic residues in α-amylase family (22). These residues are strictly conserved in the GH family 13 and corresponds to D221, E277 and D353 in DexC protein, suggesting a possible role in the hydrolysis of α-1,6 glucosidic linkages. A region located near the C-terminal of the longest loop 3 (23) is also conserved. We found that several amino acids (D77, M78, F185, Q189, N282, F304, R441, and R445) at or near the most conserved regions are strictly conserved or change to other amino acids between oligo-1,6-glucosidase, dextran glucosidase and neopullulanase subfamilies, and simultaneously they are predicted as functional residues by SequenceSpace method (see below). Those residues might explain some differences in the substrate specificity between these glucosidases. Comparative X-ray crystallographic (apo and holo forms) and site-directed mutagenesis studies would be necessary to confirm this hypothesis.

TABLE 1

BLASTX Result of the Comparison of DexC Deduced Protein to the Sequence Databases

Accession No.	Protein	Organisms	Identity (%)	E-value
AF168613	α-1-6-glucosidase	*Aspergillus parasiticus*	58	e-174
AL355920	α-glucosidase	*Schizosaccharomyces pombe*	53	e-159
S42358	α-glucosidase	*Bacillus sp.*	52	e-154
X76947	α-glucosidase	*Bacillus sp.*	52	e-154
P29094	oligo-1,6-glucosidase	*Bacillus thermoglucosidasius*	54	e-153
M94674	α-glucosidase	*Candida albicans*	47	e-141
P21332	oligo-1,6-glucosidase	*Bacillus cereus*	51	e-140
A45249	α-glucosidase MAL62	*Candida albicans*	47	e-140
P39795	trehalose-6-phosphate hydrolase	*Bacillus subtilis*	48	e-135
AF216220	α-glucosidase	*Bacillus sp. DG0303*	48	e-134
AE004175	trehalose-6-phosphate hydrolase	*Vibrio cholerae*	50	e-132
Q45101	oligo-1,6-glucosidase	*Bacillus coagulans*	48	e-132
D70034	oligo-1,6-glucosidase homolog yvdL	*Bacillus subtilis*	43	e-128
I40498	α-phosphotrehalase treA	*Bacillus subtilis*	47	e-128
D84648	exo-α-1,4-glucosidase	*Bacillus stearothermophilus*	48	e-127
P43473	α-glucosidase (maltase)	*Pediococcus pentosaceus*	46	e-127
S44188	α-glucosidase	*Staphylococcus xylosus*	45	e-125
H69755	oligo-1,6-glucosidase homolog ycdG	*Bacillus subtilis*	46	e-124
AF261762	maltase	*Pichia angusta*	42	e-122
P40884	probable α-glucosidase YJL216C	*Saccharomyces cerevisiae*	44	e-121
S66856	probable membrane protein YOL157c-yeast	*Saccharomyces cerevisiae*	43	e-121
P40439	probable α-glucosidase YIL172C/YJL221C	*Saccharomyces cerevisiae*	43	e-120
P53051	probable α-glucosidase FSP2	*Saccharomyces cerevisiae*	42	e-120
P28904	trehalose-6-phosphate hydrolase	*Escherichia coli*	41	e-119
U06195	trehalose-6-phosphate hydrolase	*Escherichia coli*	41	e-118
P29093	oligo-1,6-glucosidase	*Bacillus sp.*	44	e-117
H70011	exo-α-1,4-glucosidase homolog yugT	*Bacillus subtilis*	45	e-116
Q59905	glucan 1,6-α-glucosidase	*Streptococcus equisimilis*	44	e-112
P38158	α-glucosidase MAL3S	*Saccharomyces cerevisiae*	41	e-111
P53341	α-glucosidase MAL1S	*Saccharomyces cerevisiae*	41	e-111
P07265	α-glucosidase MAL6S	*Saccharomyces pastorianus*	41	e-110
AJ007636	maltase	*Kluyveromyces lactis*	42	e-110
Q99040	glucan 1,6-α-glucosidase	*Streptococcus mutans*	43	e-108

Note. The sequences producing significant alignments with E-value better than e-100 are shown. The percentage (%) of sequence identity is referred to the BLASTX results.

Structure Prediction of DexC

The prediction of the dextranase enzyme structure, a β-propeller fold, has been published (24) and it was include in the GH family 49 (21). The 3D structure of *B. cereus* oligo-1,6-glucosidase (PDB file:1UOK) (25) has been previously published. Based on common sequence pattern in the structural core region, between DexC and *B. cereus* oligo-1,6-glucosidase (O16G_BACCE), we generated a plausible 3D model for the DexC protein. Figure 4B shows an unambiguous alignment between DexC and O16G_BACCE due to their high degree of sequence identity (51%) even in most variable regions (VRs), and the low incidence of insertions/deletions relative to O16G_BACCE. The total number of insertions and deletions are 40 and 1, respectively. The insertion of 12 amino acids occur at the N-terminal of O16G_BACCE, 7 at the C-terminal, while others 21 insertions and the only 1 deletion occur into the core. These insertions/deletions are located at loop regions and were not modeled.

The PIRPSQ module of WHAT IF was used to build the 3D model from the predicted alignment in Fig. 4B. The average packing quality for the final model, evaluated with QUACHK module of WHAT IF, was −0.924. For comparison, a molecule is certain to be incorrect if the score is below −3.0. The superposition over equivalent 557 Cα atoms (93% of the modeled sequence) between the template structure 1UOK and the DexC model, gives a root-mean-square deviations (RMSd = 0.024 Å); while the superposition of all atoms render an RMSd value of 2.85 Å. We concluded, that the structural model, $(\beta/\alpha)_8$-barrel, of the DexC core is the correct one.

Dex (*dexA* encoded protein) and DexC proteins have not a significant sequence similarity and the predicted 3D structure is different; however, they catalyze chemically equivalent reactions (hydrolysis of the α-1,6 glucosidic linkage) on similar or identical substrates. Therefore, the structural model of DexC suggests that the enzymatic activity of α-1,6 glucosidic linkage hy-

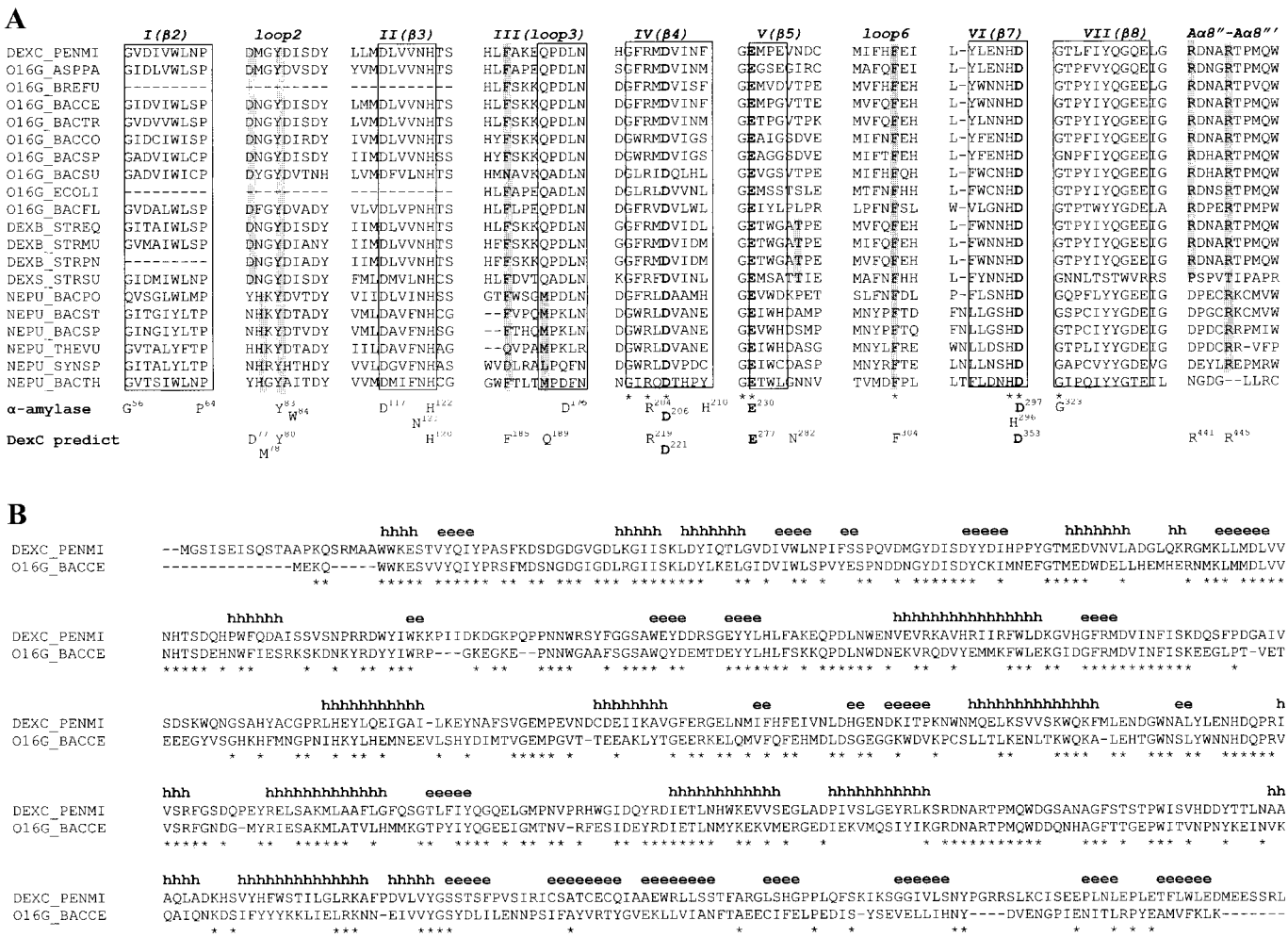

FIG. 4. (A) Alignment of the most conserved regions in oligo-1,6-glucosidases, dextran glucosidases, neopullulanases, and DexC protein. Regions conserved in the α-amylase family are in boxes. Boxes in gray show the predicted functional amino acids by SequenceSpace method and in bold are indicated the catalytic amino acids. The identical residues among these enzymes are indicated by *. Numbered amino acids correspond to catalytic or functional residues of Taka amylase and DexC predicted protein. The sequences are: DEXC_PENMI, *P. minioluteum* DexC (this study); O16G_ASPPA, *Aspergillus parasiticus* oligo-1-6-glucosidase; O16G_BREFU, *Brevibacterium fuscum* oligo-1-6-glucosidase; O16G_BACCE, *Bacillus cereus* oligo-1,6-glucosidase; O16G_BACTR, *Bacillus thermoglucosidasius* oligo-1-6-glucosidase; O16G_BACCO, *Bacillus coagulans* oligo-1-6-glucosidase; O16G_BACSP, *Bacillus sp.* oligo -1-6-glucosidase; O16G_BACSU, *Bacillus subtilis* oligo-1-6-glucosidase; O16G_ECOLI, *Escherichia coli* oligo-1-6-glucosidase; O16G_BACFL, *Bacillus flavocaldarius* oligo-1-6-glucosidase; DEXB_STREQ, *Streptococcus equisimilis* glucan 1,6-α-glucosidase; DEXB_STRMU, *Streptococcus mutans* glucan 1,6-α-glucosidase; DEXB_STRPN, *Streptococcus pneumoniae* glucan 1,6-α-glucosidase; DEXS_STRSU, *Streptococcus suis* glucan 1,6-α-glucosidase; NEPU_BACPO, *Bacillus polymyxa* neopullulanase; NEPU_BACST, *Bacillus stearothermophilus* neopullulanase; NEPU_BACSP, *Bacillus sp.* neopullulanase; NEPU_THEVU, *Thermoactinomyces vulgaris* neopullulanase; NEPU_SYNSP, *Synechocystis sp.* neopullulanase and NEPU_BACTH, *Bacteroides thetaiotaomicrom* neopullulanase. (B) Comparison between DexC and oligo-1,6-glucosidase of *B. cereus* O16G_BACCE, which known secondary structure of oligo-1,6-glucosidase as determined by DSSP program (26). Helices are indicated by h, and β-strands by e.

drolysis appears to have evolved independently on at least two structural frameworks, a $(\beta/\alpha)_8$-barrel and a β-propeller fold.

Prediction of Functional Residues for the Hydrolysis of the α-1,6 Glucosidic Linkage

The catalytic hydrolysis of the α-1,4 glucosidic linkage has been extensively studied using the α-amylase enzymes as model. For instance, D206, E230, and D297 (unless otherwise specified, all number use the Taka-amylase A numbering) (22) were recognized as catalytic residues; D117, H122, R204, and H296 involved in the active site; G56, P64, and G323 structurally important; Y83 and W84 related to transglycosylation activity; and N121, D175, and H210 constitute the calcium and chloride binding site (23).

Among all proteins with homology to DexC, we choose those which hydrolize α-1,6 glucosidic linkages (oligo-1,6-glucosidases, dextran glucosidases and neopullulanases), to predict the amino acids possibly

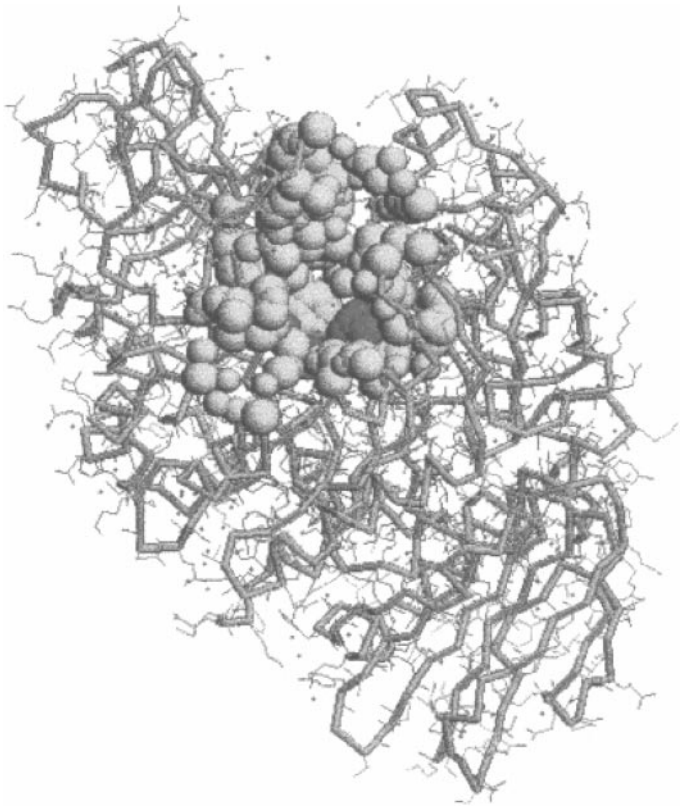

FIG. 5. Side view of the 3D structure of *B. cereus* oligo-1,6-glucosidase. Spacefill representation indicates the catalytic residues D221, E277, and D353 (dark-gray), and the predicted functional residues D77, H78, F185, M189, T282, F304, R441, and R445 (light-gray). The figure was prepared using the RasMol program (28).

involved in the α-1,6-specifity. We used a similar approach in the prediction of functional residues for the GH family 32 (27).

Based on combination of sequence analysis by SequenceSpace method, and the results of the WWW CAST service, we predicted the amino acids D77, F185, F304, R441, R445, and H, M, and T in amino acid positions corresponding to M78, Q189, N282, respectively in DexC protein, as involved in the specific hydrolysis of the α-1,6 glucosidic linkage. D77 is also conserved in maltases, threalose-6-phosphate hydrolase, and threalose synthase, but it changes to Y, H, or N in neopullulanase and some α-amylases. H78 is strictly conserved only in neopullulanases. F185, F304, and R445 are highly conserved into GH family 13. M189 is highly conserved in neopullulanase and some α-amylases, but change to Q in the remaining proteins into GH family 13. T282 is specific to dextran glucosidases, and R441 is highly conserved into the GH family 13, except, neopullulanase and some α-amylases in which it change to D or N. The localization of predicted catalytic (D221, E277 and D353: dark-gray), and functional (D77, H78, F185, M189, T282, F304, R441, and R445: light-gray) residues of DexC in the 3D structure of *B. cereus* oligo-1,6-glucosidase are shown in Fig. 5. In the present work, we have introduced a new step in order to predict functional residues. The new step is the identification of those amino acids exposed to solvent and delimiting the substrate-binding cavity, using the WWW CAST service (http://sunrise.cbs.umn.edu/cast). Then, functional residues are predicted, not only, based on pattern of amino acids conservation in subfamilies (SequenceSpace method), but also, on the solvent accessibility according to the 3D structure (WWW CAST service). This approach reduces the number of functional residues predicted by the amino acids conservation pattern.

Experiments designed to detect the functional residues in α-1,6-glucosidases are likely to be the most easily verifiable predictions of the DexC structural model we present here.

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