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The *celA* Gene, Encoding a Glycosyl Hydrolase Family 3 β-Glucosidase in *Azospirillum irakense,* Is Required for Optimal Growth on Cellobiosides

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The CelA β-glucosidase of *Azospirillum irakense,* belonging to glycosyl hydrolase family 3 (GHF3), preferentially hydrolyzes cellobiose and releases glucose units from the C_3, C_4, and C_5 oligosaccharides. The growth of a Δ*celA* mutant on these cellobiosides was affected. In *A. irakense*, the GHF3 β-glucosidases appear to be functional alternatives for the GHF1 β-glucosidases in the assimilation of β-glucosides by other bacteria.

β-Glucosidases (EC 3.2.1.21) are present in eukaryotic and prokaryotic organisms and catalyze the hydrolysis of cellobiose and chemically related β-glucosides. The β-glucosidases of cellulolytic and noncellulolytic microorganisms are key enzymes for the assimilation of cellobiose, the biodegradation product of cellulose, and other plant-derived β-glucosides, such as arbutin and salicin. These enzymes also modulate the biological activities of different β-glucosides, such as antibiotics (18) or saponins (15). Numerous β-glucosidases have been identified for cellulolytic and noncellulolytic bacteria; nevertheless, their role in plant root colonization by plant growth-promoting bacteria is not understood (14, 19, 22).

Based on their amino acid sequence similarities, β-glucosidases are classified into glycosyl hydrolase families 1 and 3 (GHF1 and GHF3, respectively) (4). The GHF3 β-glucosidases exhibit a modular organization in two domains: an N-terminal catalytic A domain and a C-terminal noncatalytic, but essential, B domain. Among the bacterial enzymes, the AB organization is the most frequent, while the BA and AB′ (exhibiting a truncated C-terminal domain referred to as B′) types have also been described (7, 13, 31). The functional analysis of GHF3 β-glucosidases using genetic approaches has been documented for only a few bacteria (7, 18, 31, 33).

Azospirillum bacteria colonize the rhizosphere of several gramineous plants, such as rice, maize, sorgo, and wheat (17, 28, 29). The ability of *Azospirillum irakense* to grow on pectins and plant-derived β-glucosides, such as cellobiose, arbutin, and salicin, has been reported (2, 7, 11, 12). The isolation and characterization of the corresponding enzymes could reveal potential commercial applications (e.g., with respect to specific activities, substrate specificities, stability, and so forth). Two GHF3 β-glucosidases, SalA and SalB, are required for the growth of *A. irakense* on salicin (7). In this work, we describe the characterization of a third GHF3 β-glucosidase, CelA, for *A. irakense* KBC1. The encoding *celA* gene is required for optimal growth on cellobiose and cellulose-derived oligosaccharides, emphasizing the importance of the GHF3 enzymes in the assimilation of plant-derived β-glucosides, a feature which is usually assigned to GHF1 enzymes.

Identification of the *celA* locus. Approximately 3,000 *Escherichia coli* clones from a genomic library of *A. irakense* KBC1 (2) were tested for β-glucosidase (with methylumbelliferyl-β-glucuronide [MUG] as a substrate) and endoglucanase (with carboxymethyl cellulose as a substrate) activities with overlay methods (7, 19). No clone exhibiting endoglucanase activity was obtained. The MUG-positive clones were classified into two families, based on the restriction pattern of their cosmids with *Eco*RI, *Hin*dIII, and *Bam*HI. From each family, one cosmid was retained for further characterization. pFAJ0650, harboring two β-glucosidases, SalA and SalB, has been previously described (7); cosmid pFAJ0649 was analyzed in this work (Table 1). The cosmid insert DNA contained five *Eco*RI restriction fragments, of 8, 5.5, 4.5, 3.2, and 1.5 kb. Each of these restriction fragments was subcloned into cloning vector pBluescript SK(−). Clones showing MUG activity were obtained only for the 5.5-kb fragment (pFAJ0680). The putative gene encoding β-glucosidase activity was named *celA*.

Sequence analysis of the *celA* gene. DNA sequencing of pUC18/19 or SK(−) subclones was carried out with an AutoRead sequencing kit (Pharmacia-LKB) and an automated sequencer (ALF; Pharmacia-KLB). Sequence data were processed and analyzed by using the PCGene software package (Intelligenetics). We sequenced, on both strands, 2.5 kb of plasmid pFAJ0680 and found an open reading frame encoding 685 amino acids with a predicted molecular mass of 73 kDa. The N-terminal region of this deduced amino acid sequence exhibited a putative peptide signal 22 residues long: MGAL RLLGSISIVALTCGGIHA/STAIAQE (the slash indicates the postulated cleavage site). This prediction of the amino-terminal signal sequence was obtained with the SignalpWWW Server (http://www.cbs.dtu.dk/services/SignalP/) (16).

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TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Reference or source
Strains		
A. irakense		
KBC1	Wild type; LMG 10653; isolated from rice rhizosphere	11
FAJ0694	KBC1 *celA*::*kan* Km^r	This work
E. coli DH5α	F80*dlacZ*ΔM15 Δ(*lacZYA-argF*) *recA endA hsdr supE*	Gibco BRL
Plasmids		
pUC19	Cloning vector; Ap^r	34
pBluescript SK(−)	Cloning vector; Ap^r	Stratagene
pSUP202	Mobilizable plasmid; suicide vector for *A. irakense*; Cm^r Tc^r Ap^r	24
pRK2013	Tra$^+$ helper plasmid	9
pLAFR1	IncP1 broad-host-range plasmid;Tc^r	10
pLAFR3	pLAFR1 derivative containing the pUC8 multiple cloning site	26
pHP45Ω-Km	Tc^r; contains Km^r cassette	8
pFAJ0649	pLAFR1 derivative from genomic library of *A. irakense* containing *celA*	This work
pFAJ0680	pBluescript SK(−) with 5.5-kb *Eco*RI insert from pFAJ0649; expresses CelA	This work

A comparison of the deduced sequence with sequences in data banks revealed similarity with the GHF3 β-glucosidases (Table 2). The classification of glycosyl hydrolases is available at the following address (4): http://afmb.cnrs-mrs.fr/~pedro/CAZY/db.html. CelD of *Pseudomonas fluorescens* showed a particularly high degree of identity with CelA (43.6%), while a lower degree of identity was found between CelD and SalA (24.2%) or SalB (20.5%) and between CelA and SalA (23.4%) or SalB (22.9%). No match was obtained with the GHF1 β-glucosidases. The CelA sequence has all the characteristics of the AB enzymes: an N-terminal A domain with a conserved putative catalytic aspartate residue and a C-terminal B domain showing the typical conserved regions which are also present in the AB or AB′ enzymes (Table 2). *A. irakense* is the first bacterium for which three orthologous GHF3 β-glucosidases have been described.

Biochemical characteristics of CelA. A CelA extract was prepared from *E. coli* harboring plasmid pFAJ0680. Cells of overnight cultures were collected by centrifugation, washed in phosphate buffer (0.1 M K_2HPO_4-KH_2PO_4 [pH 7.0]), and lysed by sonication. The lysates were cleared by centrifugation, stored at −20°C, and analyzed for β-glucosidase activity. No β-glucosidase activity was detected in *E. coli* with the SK(−) plasmid. Moreover, the insertion of a Km^r cassette into the *celA* gene abolished the expressed β-glucosidase activity in *E. coli* (see below). We also verified that a unique signal was present in the zymogram of the crude CelA extract (data not shown). These features suggested that the β-glucosidase enzyme expressed from plasmid pFAJ0680 is encoded by *celA*.

The K_m and specific activity of the CelA extract were measured with *p*-nitrophenyl-β-D-glucoside (PNPG), *p*-nitrophenyl-β-D-xyloside (PNPX), cellobiose, gentiobiose, and salicin as substrates. The colorimetric methods used have been described previously (7). The optimal conditions retained for β-glucosidase activity were pH 7 and 45°C. The extract from *E. coli*/pFAJ0680 did not hydrolyze PNPX. The K_m determination revealed a greater affinity for cellobiose than for gentiobiose or the aryl-β-glucosides salicin and PNPG (Table 3). In the presence of 10 mM salicin, gentiobiose, or PNPG, the specific activity ranged from 10 to 35% of the optimal activity obtained with cellobiose. The CelA extract also exhibited the capacity to hydrolyze cellotriose, cellotetraose, and cellopentaose; nevertheless, optimal activity was obtained with cellobiose as a substrate (Table 3). This additional hydrolytic activity of CelA suggests an exo-1,4-β-glucosidase activity (EC 3.2.1.74), which is also exhibited by other GHF3 enzymes, such as CelD of *P.*

TABLE 2. Comparison of the deduced amino acid sequences of CelA with the sequences of some bacterial GHF3 β-glucosidases

β-Glucosidase	Species	Origin of the sequences (reference)	% Identity with CelA[a]	Active-site homology[b]
CelA	*A. irakense*	This work	100.0	306 HKQL**L**TDV**L**KGQMGFN**G**FIVG**D**WNAHDQVPGC
CelD	*P. fluorescens*	20	43.6	320 HKYL**L**TDV**L**KDLSGFD**G**LVVG**D**WSGHSFIPGC
SalA	*A. irakense*	7	23.4	233 NDHL**L**NKV**L**KGDWGYK**G**WVMS**D**WGAVPATDFA
OleR	*Streptomyces antibioticus*	18	20.9	193 SDEL**L**NKV**L**KEQWKFR**G**WVTS**D**WLATQSTDAL
Cbg1	*Agrobacterium tumefaciens*	3	21.6	201 NPWL**L**TKV**L**REEWGFD**G**VVMS**D**WFGSHSTAET
BglX	*E. coli*	33	27.5	246 DSWL**L**KDV**L**RDQWGFK**G**ITVS**D**HGAIKELIKH
BgxA	*E. chrysanthemi*	31	21.5	314 NRFL**L**TDL**L**RGQYGFD**G**VILS**D**WLITNDCKGD
SalB	*A. irakense*	7	22.9	313 NKQM**L**IDL**L**RGTHKFK**G**LILS**D**WAITNDCNES

[a] The putative signal sequences were removed from the analyzed sequences. The percentages of identity were determined by using the ALIGN program of the GENESTREAM network server (http://vega.igh.cnrs.fr) at the Institut de Génétique Humaine Center (Montpellier, France).

[b] The putative catalytic residue, which was experimentally identified for *Aspergillus wentii* (1), is underlined in all the sequences. Boldfacing indicates conserved amino acid residues.

TABLE 3. Substrate specificity of CelA[a]

Substrates	K_m (mM)	Sp act[b]	% Relative activity
β-Glucosides			
Cellobiose	0.05	386.0	100
Gentiobiose	0.26	82.9	21
Salicin	0.24	38.8	10
PNPG	1.33	138.0	35
Cellobiosides			
Cellobiose		449.2	100
Cellotriose		171.7	38
Cellotetraose		168.0	37
Cellopentaose		153.4	34

[a] Two classes of substrates, β-glucosides and cellobiosides, were used; in each set of experiments, cellobiose was used as a reference. Because of differences in experimental procedures, the absolute specific activities between the two experiments cannot be compared.

[b] Micromoles of glucose or *p*-nitrophenol produced per minute per milligram of protein.

fluorescens subsp. *cellulosa* (20) and CdxA of *Prevotella ruminicola* (32). Despite this secondary activity, the main characteristic of CelA is the hydrolysis of cellobiose, and the enzyme can therefore be referred to as a cellobiase type. In *A. irakense* KBC1, the previously characterized adjacent *salA* and *salB* genes were implied to be involved in salicin assimilation, and their encoding β-glucosidases exhibited more efficient hydrolysis of aryl-β-glucosides than of cellobiose (7). Moreover, the *salCAB* operon of *A. irakense* is specifically induced by aryl-β-glucosides and not by cellobiose (25). These features suggest a specialization of the three GHF3 β-glucosidases in two different assimilatory pathways for either the aryl-β-glucosides or the cellulose-derived oligosaccharides.

Construction and phenotype of a Δ*celA A. irakense* mutant. Plasmid pFAJ0680 was digested with *Bss*HII, blunt ended, and ligated with the blunt-ended Km^r cassette isolated from pHP45Ω-Km (Table 1). The resulting plasmid did not express β-glucosidase activity in *E. coli* transformants. This constructed deletion was subcloned, as an *Eco*RI fragment, into vector pSUP202. Triparental mating with the pRK2013 helper plasmid allowed the transfer of the pSUP202 derivative into *A. irakense* KBC1. Km^r *A. irakense* mutants were isolated and checked by hybridization for double homologous recombination. The constructed *A. irakense* mutant was named FAJ0694 (Fig. 1), and its growth on MMAB minimal medium (30) supplemented with different sugars as sole carbon sources was compared with that of the wild type. Because only small quantities of C_3, C_4, and C_5 cellobiosides were available, all growth curves were monitored by use of microplates with 200 μl of minimal medium.

Unlike wild-type *A. irakense*, FAJ0694 exhibited strongly delayed growth on C_2, C_3, C_4, and C_5 cellobiosides. Wild-type *A. irakense* cultures reached an optical density at 595 nm of 0.5 within 14 h, while FAJ0694 required 24 h to reach the same optical density at 595 nm for each of the cellobiosides tested. The growth of FAJ0694 on the aryl-β-glucosides arbutin and salicin or on glucose or malate was not affected. This phenotype is in agreement with the substrate specificity of the CelA enzyme (Table 3). While *A. irakense* uses these oligosaccharides as sole sources of carbon, it does not assimilate carboxymethyl cellulose and cannot be regarded as a truly cellulolytic bacterium. However, because the constructed Δ*celA* mutant reached the same cell density as the wild type on cellobiosides after 24 h of culturing and because the *salCAB* operon was not induced by cellobiose (25), it is likely that as-yet-unidentified alternative β-glucosidases support the growth of *A. irakense* on cellobiosides.

GHF3 β-glucosidases are alternatives for GHF1 β-glucosidases in *A. irakense*. The assimilatory pathways of β-glucosides are well characterized for *E. coli* (23) and *Erwinia chrysanthemi* (5, 6). All the β-glucosidases of these pathways belong to GHF1 (4), and their homologs have been identified for both gram-positive and gram-negative bacteria (21). *A. irakense* has been shown to utilize a β-glucoside assimilatory pathway involving at least three GHF3 β-glucosidases, SalA, SalB, and CelA. The GHF3 β-glucosidases seem to be functional alternatives for GHF1 β-glucosidases in the assimilation of β-glucosides, such as salicin and cellobiose, in *A. irakense*. Moreover, the organization and the regulation of the *salCAB* operon also suggest that the assimilation of aryl-β-glucosides occurs via an original mechanism which has been not described for other gram-positive or gram-negative bacteria (25).

This work also contributed to the functional analysis of one of the recently described clusters of orthologous genes (COG), COG2091, which matches the GHF3 genes (27). One goal of the COG databases is to facilitate the assignment of a function to the deduced open reading frames of a sequenced genome. Therefore, knowledge of the physiological role of several β-glucosidases of this GHF3-COG2091 cluster is necessary for

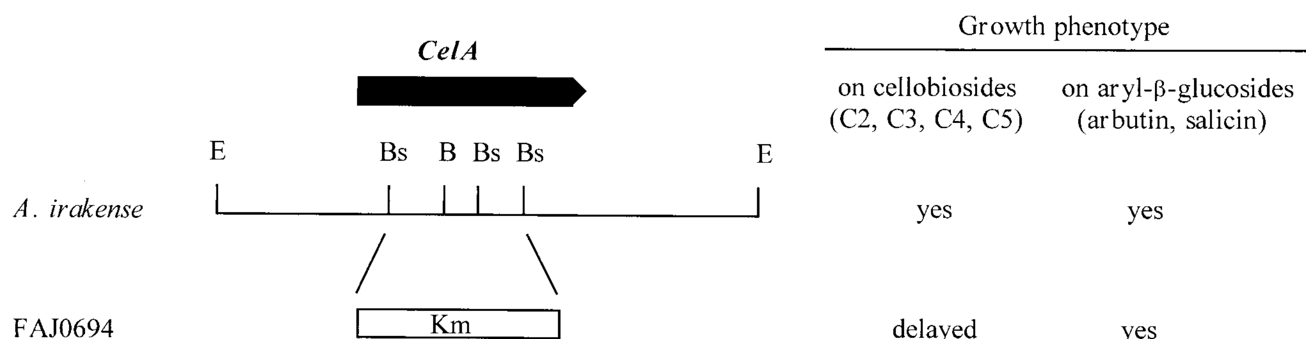

FIG. 1. Construction and phenotype of the Δ*celA A. irakense* mutant. E, *Eco*RI; Bs, *Bss*HII; B, *Bam*HI.

further predictive investigations. In addition, because three orthologous β-glucosidases, CelA, SalA, and SalB, are present in the same bacterium, *A. irakense* KBC1, the phylogenetic relationships among the members of this GHF3-COG2091 cluster must be clarified.

Nucleotide sequence accession number. The sequence obtained in this study was submitted to the GenBank database under accession number AF213463.

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