AEM Accepts, published online ahead of print on 18 May 2007
Appl. Environ. Microbiol. doi:10.1128/AEM.00496-07


**Short communication**

# Alpha-agarases define a new family of glycoside hydrolases, distinct from beta-agarase families

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Keywords: marine bacteria, agar, glycoside hydrolase, CBM6, calcium-binding module

Running title: Alpha-agarases define a new family of glycoside hydrolases

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Abbreviations: aa, amino acid(s); bp, base pair(s); kb, kilobase(s); nt, nucleotide, kDa, kiloDalton; CBM(s), carbohydrate-binding module(s); GH(s), glycoside hydrolase(s); SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, ORF, open reading frame; TSP3, thrombospondin type 3 repeats.

**ABSTRACT**

The gene of the α-agarase from "*Alteromonas agarilytica*" has been cloned and sequenced. The gene-product (154 kDa) is unrelated to β-agarases and instead belongs to a new family of glycoside hydrolases (GH96). The α-agarase also displays a complex modularity, with the presence of five TSP3 repeats and three CBM6s.

Agars are the main cell wall components of numerous red macroalgae. These polymers consist of 3,6-anhydro-L-galactoses and D-galactoses alternatively linked by α-(1,3) and β-(1,4) linkages (10). Agars constitute a crucial carbon source for a number of marine bacteria which secrete agarolytic enzymes, mainly β-agarases (EC 3.2.1.81) which hydrolyze the β-(1,4) linkages (See for a review, 12). They are found in three distinct families of glycoside hydrolases, families GH16, GH50 and GH86 (CAZY database, 6). Structural data are only available for the GH16 β-agarases *Zg*AgaA and *Zg*AgaB from *Zobellia galactanivorans* (1, 2, 9). In contrast, *"Alteromonas agarilytica"* secretes an α-agarase (EC 3.2.1.158) which randomly hydrolyzes the α-(1,3) linkages in agars, releasing agarotetraose as its main end product (13, 15, 18). In this context, we sought to determine whether the agarases of the α-type share any structural relationships with β-agarases. We report here the cloning of the α-agarase gene *agaA* from "*A. agarilytica"* and we demonstrate that α-agarases define a new GH family.

**Cloning of the *agaA* gene.**

The α-agarase from "*A. agarilytica"* (*Aa*AgaA) was purified as previously described (13) and N-terminal and internal peptide sequences were determined (Pasteur Institute, France): ETLELQAESFANSGG (A) and QPRVYNPNEHIVAEIQGPAT (B), respectively. With degenerated oligonucleotides derived from these microsequences (CARGCIGARTCITTYGCIAA and TAYAAYCCNAAYGARCAYAT, respectively), a 2.5 kb DNA fragment was amplified by PCR using *A. agarilytica* genomic DNA and labeled with [α-$^{32}$P]dCTP. This radiolabeled probe was used to screen an "*A. agarilytica"* genomic library, prepared as previously described (5). Among the ~5000 recombinant clones, two positive clones (pAA1 and pAA2) were identified with inserts of 7.4 kb and 17.9 kb in length. Southern blot analysis and plasmid mapping indicated that both inserts encompassed the same gene which is present in only one copy in the genome. Plasmid pAA1 was sequenced on both strands over 4651 bp and a single ORF

was identified and referred to as *agaA* (4287 bp). Potential -35 and -10 promoter regions (TTGAtc and TAcAca) and a Shine Dalgarno sequence (GGAG) were identified upstream of the start codon. A possible transcription termination codon was found downstream of the TAA stop codon. The deduced gene-product is a preprotein of 1429 residues (154 kDa) which includes the peptides A and B. A signal peptide cleaved between A26 and E27 is predicted by SIGNALP (4), consistent with the N-terminal sequencing of the purified extracellular α-agarase.

Several attempts were made to overexpress *Aa*AgaA and its isolated modules in *E. coli* with pET or pGEX vectors in various conditions. Unfortunately, the constructs always yielded inclusion bodies. However, within one week of culture at 22°C on Zd agar broth (3), the *E. coli* clones harboring the plasmid pAA1 dug a hole in the substratum, indicating agar degradation. Therefore, under the control of its own promoter, *agaA* was successfully translated into an active, recombinant enzyme, confirming that this gene indeed encodes the α-agarase.

**_Aa_AgaA is a complex, modular protein with a catalytic domain defining a new GH family**

Only the N-terminal region of *Aa*AgaA displays significant sequence similarity with proteins in the UniProt database. Based on InterProScan (14), eight distinct modules were identified in this region (Fig. 1A), five thrombospondin type 3 repeats (TSP3-1, D171-G203; TSP3-2, D360-L392; TSP3-3, D393-A425; TSP3-4, D426-L458; TSP3-5, D459-G491) and three carbohydrate-binding modules from the family 6 (CBM6-1, E27-R159; CBM6-2, E209-T343; CBM6-3, S659-L792).

The closest characterized protein matching the TSP3 repeats of *Aa*AgaA is the cellulase CelG from *Pseudoalteromonas haloplanktis*. In CelG, the TSP3 repeats constitute an extended linker connecting the GH5 catalytic module and a C-terminal CBM5 (17). The TSP3

repeats of *Aa*AgaA present about 50% sequence identity with their counterparts in CelG. They also display ~30% sequence identity with the "true" type 3 repeats found in human thrombospondin, whose crystal structure has been solved (11). These modules lack secondary structures and are organized around a core of calcium ions coordinated by conserved aspartates (DxDxDGxx[D/N]xxDxC motif). The conserved cysteine is involved in a disulphide bridge linking adjacent TSP3 repeats, strengthening their stability (11). This motif is strictly conserved in each of the TSP3 repeats of *Aa*AgaA (Fig. 1B), indicating that these modules adopt similar structure and likely bind calcium ions. This is consistent with the observations that α-agarase activity is stabilized by the presence of calcium ions (13, 18).

In BLASTp searches with the three CBM6s from *Aa*AgaA, the highest E-values are always obtained for the CBM6 sequences tethered to β-agarases, while they significantly decrease with CBM6 linked to non-agarolytic enzymes. Only the CBM6s attached to the β-agarases *Sd*Aga16B and *Sd*Aga86E from *Saccharophagus degradan*s were shown to actually bind agarose (8). A pairwise comparison indicates a strong sequence identity (51%) of CBM6-1 with the CBM6 from *Sd*Aga16B (Fig. 1C), while CBM6-2 and CBM6-3 are more divergent (28% and 26%, respectively). The crystal structure of the CBM6 from *Sd*Aga16B revealed that five residues are critical for the recognition of the non-reducing end of the agarose chain: Asn39, Tyr40, Trp97, Trp127 and Asn130 (8). Four of these residues are strictly conserved in CBM6-1, while Tyr40 is substituted by a similar aromatic amino acid, Phe64 (Fig. 1C). Altogether these results strongly suggests that CBM6-1 is an agar-binding module and likely displays selectivity towards the non reducing termini of agarose chains. In contrast, the five critical residues are only partially conserved in CBM6-2 and CBM6-3. Therefore the specificity of these latter CBM6 is less certain.

Finally, a BLASTp search on the patent databank at the NCBI identified two proteins from a marine bacterium with a strong sequence identity with the C-terminal region of

*Aa*AgaA (49% and 77% respectively, Fig. 2). These proteins, also described as α-agarases (16), encompass three modules, two N-terminal CBM6s and the C-terminal module conserved with *Aa*AgaA. Since the N-terminal region of *Aa*AgaA encompasses only additional, non-catalytic modules, its conserved C-terminal region (Asn809-His1429) likely contains its active site. Together these three catalytic modules (~620 residues) constitute a new family of glycoside hydrolases, referred to as family GH96 (CAZY database). Therefore α-agarases are structurally unrelated to the β-agarases from the families GH16, GH50 and GH86.

## ACCESSION NUMBERS

The nucleotide sequence of the α-agarase from "*Alteromonas agarilytica"* has been deposited in Genbank with the accession number AF121273. Its amino acid sequence is available in Swiss-Prot under the accession number Q9LAP7.


## ACKNOWLEDGMENTS

The authors gratefully acknowledge the help of the Conseil Régional de Bretagne, which supported this research through a Ph.D. fellowship to D. F. and a research grant (PRIR n° 98C389).

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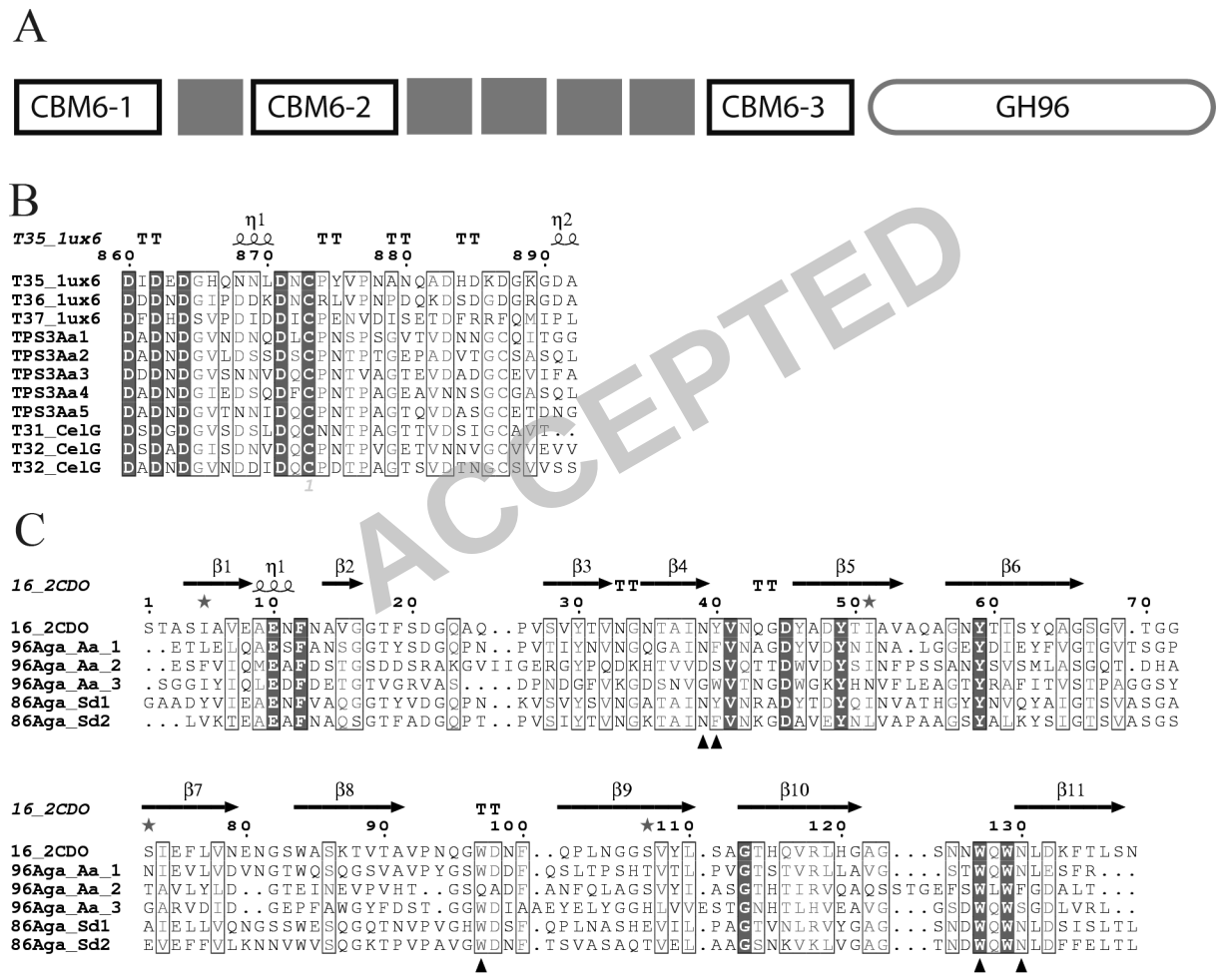
## FIGURE LEGENDS

**Figure 1: A**. Modular architecture of the α-agarase *Aa*AgaA. CBM6 and GH96 refer to carbohydrate-binding modules of the family 6 and glycoside hydrolase module of the family 96, respectively. The grey boxes correspond to the thrombospondin type 3 repeats (TSP3). **B.** Structure-based alignment of the TSP3 repeats of the human thrombospondin (PDB code 1UX6), of the α-agarase *Aa*AgaA and of the cellulase CelG from *Pseudoalteromonas haloplanktis* (trEMBL code: O86099). **C**. Structure-based alignment of the three CBM6s from the α-agarase *Aa*AgaA and of the agar-specific CBM6s tethered to the β-agarases *Sd*Aga16B (Genpept: ABD80437) and *Sd*Aga86E (Genpept: ABD81915) from *Saccharophagus degradans*. These modules are compared to the secondary structures of the CBM6 appended to *Sd*Aga16B (PDB code 2CDO). Alpha helices and beta strands are represented as helices and arrows, respectively, and beta turns are marked with TT. The black triangles mark the residues involved in the recognition of the non-reducing end of agarose chains. Figure 1B, C and Figure 2 were prepared using the program ESPrit (7) and use the same colour codes.

**Figure 2:** Sequence alignment of the catalytic module of AgaA from *A. agarilytica* and of Aga14 and Aga15 (Patent US 6599729). *Dark shaded boxes* enclose conserved positions.


A
CBM6-1
CBM6-2
CBM6-3
GH96
B
T35_1ux6
η1
η2
860
870
880
890
T35_1ux6
T36_1ux6
T37_1ux6
TPS3Aa1
TPS3Aa2
TPS3Aa3
TPS3Aa4
TPS3Aa5
T31_CelG
T32_CelG
T32_CelG
C
16_2CDO
β1
η1
β2
β3
β4
β5
β6
16_2CDO
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96Aga_Aa_3
96Aga_Sd1
86Aga_Sd2
β7
β8
β9
β10
β11
16_2CDO
96Aga_Aa_1
96Aga_Aa_2
96Aga_Aa_3
86Aga_Sd1
86Aga_Sd2


A-aga_Aa.
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