

Phylogenomic and multi-omics analysis of *Agarivorans* sp. D1326 uncovers an agarolytic pathway involving rare GH118 β -agarase

Tao Li

Henan Medical University

Cong Liu

Henan Medical University

Chengwei Wu

Henan Medical University

Boyu Zhang

Henan Medical University

Ziwei Wang

Henan Medical University

Yueyue Zhao

Henan Medical University

Wenbin Gu

Henan Medical University

Zilin Liu

Henan Medical University

Fanling Meng

Henan Medical University

Jingjing Shangguan

Henan Medical University

Wenbo Zhang

Henan Medical University

Xueyi Zhu

Zhengzhou Feier Medical Laboratory Co., LTD

Yan Wang

wangyan19820527@xxmu.edu.cn

Henan Medical University

Keywords: Marine bacterium, GH118 β -agarase, Agarivorans, Agarolytic pathway, Agar biorefinery, Multi-omics

Posted Date: July 29th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-9391058/v1>

License:  This work is licensed under a Creative Commons Attribution 4.0 International License.
[Read Full License](#)

Additional Declarations: No competing interests reported.

Abstract

Agar, a key polysaccharide in marine red algae, offers a sustainable biomass resource. In this study, phylogenetic and phylogenomic analyses classified the agarolytic marine bacterium strain D1326^T, isolated from the Yellow Sea, as a member of the genus *Agarivorans*. This work elucidates the comprehensive agarolytic network of *Agarivorans* sp D1326 by multi-omics. Genomic analysis revealed a diverse repertoire of agarase genes, including glycoside hydrolase (GH) members from the GH16, GH50, GH86, and GH118 families. Transcriptomic and proteomic profilings demonstrated the strong co-upregulation and predominant extracellular secretion of these enzymes upon agarose induction. Three key secreted β -agarases—GH86 (Aga1002), GH118 (Aga2820), and GH16 (Aga2799)—were identified as primary initiators, showing extraordinary extracellular upregulation (443.97-fold, 303.7-fold, and 299.69-fold, respectively) and likely establishing distinct pathways for producing various neoagarooligosaccharides. The initial cleavage by the Aga2820 into neoagarooctaose/decaose, which then serve as substrates for further degradation by GH16, GH50, and GH86 agarases. GH50-family enzymes, exhibiting dual localization, facilitate supplementary degradation both outside and inside the cell. A notable finding is the extracellular detection of key metabolic enzymes—neoagarobiose hydrolase and the AHG-dehydrogenase/cycloisomerase—despite their lack of signal peptides. This suggests non-classical secretion and points to a previously unrecognized capacity for partial extracellular processing of AHG into 3,6-anhydro-L-galactonate, a central metabolic intermediate. The proposed agarolytic pathway integrates extracellular polysaccharide depolymerization, oligosaccharide transport, and intra- and extracellular sugar metabolism. This work provides the first comprehensive elucidation of a native bacterial agarolytic network incorporating a rare GH118 β -agarase, offering fundamental insights for the enzymatic valorization of agar.

Introduction

Macroalgae are key primary producers in marine ecosystems and support diverse microbial communities by providing substantial nutrient input [1, 2]. In the context of sustainable development and depleting petrochemical resources, algal biomass has garnered significant interest as a promising renewable feedstock. Unlike terrestrial lignocellulosic biomass, marine macroalgal biomass grows rapidly and is lignin-free, which facilitates its conversion into valuable products [3]. A major component of this biomass is cell wall polysaccharides, which vary by algal type: agar, carrageenan, and porphyran in red algae; fucoidan in brown algae; and ulvan in green algae [4–6]. However, despite their ecological and potential industrial importance, the molecular mechanisms underlying the microbial degradation and utilization of these complex marine polysaccharides remain poorly understood, especially compared to the well-characterized systems for terrestrial plant polysaccharides like cellulose and xylan.

The rapid and efficient bioconversion of algal polysaccharides (e.g. agar) in the ocean constitutes a fundamental biochemical process driving nutrient cycling in the biosphere [7]. Reflecting this ecological role, marine ecosystems harbor the largest reservoir of agarolytic genes. Bacteria specialized in algal polysaccharide conversion typically possess genomes enriched with carbohydrate-active enzyme

(CAZyme) genes from hallmark agarase glycoside hydrolase (GH) families. According to the CAZy database, β -agarases are classified into families GH16, GH50, GH86, and GH118, while α -agarases belong to GH96 [8]. Among these β -agarase families, GH118 has the fewest members, especially when compared to the highly abundant GH16 family [9]. To date, only four GH118 β -agarases have been characterized from marine microorganisms: AgaXa from *Catenovulum* sp. X3 [10], AgaB from *Pseudoalteromonas* sp. CY24 [11], AgaC from *Vibrio* sp. PO-303 [12], and AgaJA2 from *Agarivorans* sp. JA-1 [13]. This scarcity underscores the limited exploration of this family. Furthermore, GH118 agarases exhibit distinct product profiles. While GH16 enzymes predominantly yield neoagarotetraose (NA4) and neoagarohexaose (NA6), and GH50/GH86 primarily produce neoagarobiose (NA2) [14], GH118 members typically generate oligosaccharides with higher degrees of polymerization. For instance, AgaXa produces NA6, neoagarooctaose (NA8), neoagarodecaose (NA10), and neoagarododecaose (NA12) [10], and the GH118 enzyme from *Pseudoalteromonas* sp. CY24 mainly yields NA8 and NA10 [11].

Microorganisms capable of agar degradation typically employ multi-enzyme systems comprising agarases from different GH families, ensuring efficient carbon acquisition [15]. Genomic, proteomic, and biochemical studies have elucidated these complex agarolytic pathways in various bacteria, revealing intricate enzymatic synergy. For instance, *Zobellia galactanivorans* utilizes a suite of GH16 β -agarases (AgaA, AgaB, AgaC, and AgaD) and α -porphyranases for synergistic degradation of both neutral and sulfated agar polymers [15]. Similarly, *Vibrio* sp. strain EJY3 employs four GH50 β -agarases and an α -neoagarooligosaccharide (α -NAOS) hydrolase to process agarose via two distinct pathways [16]. In *Saccharophagus degradans* 2–40, a combination of GH16, GH50, and GH86 agarases with endo- and exo-lytic activities constitutes an efficient degradation system [17]. Notably, agarases (e.g., from GH16, GH50, GH86 families) often exhibit broad substrate specificity. They can not only degrade agarose but also further cleave shorter NAOS, a key feature for pathway synergy [18]. For example, the novel GH50 β -agarase AgWH50B, isolated from *A. gilvus* WH0801, degrades both NA6 and NA8 into NA4 [19]. The GH16 family β -agarase GH16B, from *Cellvibrio* sp. KY-GH-1, catalyzes the degradation of longer-chain neoagarooligosaccharides (NA8, NA10, and NA12) to yield the final products NA4 and NA6 [20]. A GH86 agarase from *B. uniformis* NP1 digests NA8 and NA6 to NA2 [21]. Given the above findings, it is reasonable to speculate that in microorganisms possessing GH118 agarases, these enzymes play a crucial role in agarose degradation. They likely initiate the process by cleaving agarose into NAOS with a high DP. These intermediate oligosaccharides could then serve as preferential substrates for other agarase families (e.g., GH16, GH50, GH86), facilitating a synergistic, multi-enzyme cascade that enables efficient and rapid depolymerization. While the specific role and integration of the rare GH118 family within complete agarolytic pathways remain unclear, highlighting a critical gap and a promising direction for future research.

In this study, a marine agarolytic bacterium, *Agarivorans* sp. D1326, was isolated from a seaweed sample. Genomic analysis revealed a rare GH118 family agarase (Aga2820) and a diverse repertoire of agarases belonging to families GH16, GH50, and GH86, with multiple isoforms present in several families. Integrated transcriptomic and proteomic analyses were employed to elucidate the agar degradation pathway, delineating the specific roles of Aga2820 and other agarases. These findings

provide mechanistic insights into multi-agarase synergy in *Agarivorans* sp. D1326, establish a foundation for comparative studies of agarolytic systems across marine bacteria, and expand the repertoire of enzymatic resources for functional oligosaccharide production.

Materials and methods

Materials

Bacto peptone and yeast extract were purchased from Thermo Fisher Scientific Inc (USA). Other reagents were supplied by Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China).

Environmental sampling and strain isolation

A marine algal specimen was collected from a coastal site in the Yellow Sea, China (121°27'46"E, 37°28'15"N). For bacterial isolation, one gram of the alga was rinsed three times, homogenized in 3 mL of sterile saline solution (0.85% w/v NaCl), and the resultant homogenate was inoculated onto marine agar 2216 (MA; Becton Dickinson) plates with subsequent incubation at 28°C. Agarolytic activity was indicated by halo formation around colonies, which were subsequently purified via three consecutive streaking procedures on MA. Purified isolates were cultured on MA at 28°C and maintained as glycerol stocks (15% v/v) in sterile saline at – 80°C for long-term storage.

Phenotypic characterizations

Gram staining were performed using previously reported methods [22]. The salinity range supporting growth of the strain was evaluated using a modified synthetic marine ZoBell broth, consisting of 5 g Bacto peptone, 1 g yeast extract, and 0.1 g FePO₄ per liter of modified artificial seawater, supplemented with NaCl at concentrations ranging from 0 to 10% (w/v) in 1% increments. In this modified artificial seawater, all sodium ions were replaced with equivalent potassium ions. Growth temperature was assessed by incubating the strain in Marine Broth (MB; Becton Dickinson) at 8, 12, 16, 20, 24, 28, and 32°C for two days, and at 0, 4, 37, 42, and 45°C for 14 days. The pH range for growth was tested in MB adjusted to pH values from 2.0 to 10.0, using citrate–phosphate buffer (pH 2.0–7.0), Tris–HCl buffer (pH 8.0–9.0), or sodium carbonate–sodium bicarbonate buffer (pH 10.0). Physiological characteristics were examined using API 20E strips after growth on Marine Agar (MA) at 28°C for 48 hours. All procedures followed the manufacturer's instructions, except for inoculum preparation, where sterile artificial seawater was used instead of the recommended suspension medium.

Analysis of 16S rRNA gene-based phylogeny

Genomic DNA extracted from strain D1326^T using a Bacterial Genomic DNA Isolation Kit (Shanghai Sangon, China) served as template for 16S rRNA gene amplification. PCR was conducted with universal

bacterial primers B8F and B1510 (Table S1). The amplified product was gel-purified with a TaKaRa Agarose Gel DNA Purification Kit (TaKaRa, China) and ligated into the pMD™19 vector (TaKaRa, China). Following transformation, plasmids containing the correct insert were sequenced by Shanghai Sangon (China) using an ABI3730 DNA analyzer (Applied BioSystems). The obtained 16S rRNA gene sequence was submitted to GenBank and compared against the NCBI database via BLAST. The EZBioCloud platform (<https://www.ezbiocloud.net/identify>) was employed to identify phylogenetic neighbors and calculate sequence similarities [23]. Sequences used for phylogenetic analysis were aligned with the MUSCLE program [24]. The maximum-likelihood method implemented in MEGA version 11.0.13 [25] was employed to reconstruct phylogenetic trees based on 16S rRNA gene sequences, and bootstrap values were calculated from 1000 replicates.

Genome sequencing and phylogenomic analysis

The complete genome of strain D1326^T was sequenced using the paired-end method on an Illumina HiSeq X Ten platform at Novogene Company (Beijing, China). After quality filtering of the raw reads, high-quality paired-end reads were assembled de novo using SOAPdenovo version 2.04 [26]. Detailed methods for genome annotation and phylogenomic analysis are provided in the supplementary information.

A genome-based phylogenomic analysis was performed using the Genome Taxonomy Database Toolkit (GTDB-Tk v2.4.0), which based on the concatenated protein sequences of 120 ubiquitous single-copy marker genes (the bac120 set from GTDB Release 220) [27]. MEGA 11.0.13 was employed to align these joined amino acid sequences and to reconstruct a maximum-likelihood tree, with bootstrap support evaluated from 1,000 replicates.

The Average Nucleotides Identities (ANI) values were conducted using Enveomics Gateway (<https://enveomics.scigap.org/>). The in silico DNA–DNA hybridization (DDH) values were calculated using the Genome-to-Genome Distance Calculator (GGDC) 3.0, available via the Type (Strain) Genome Server (TYGS, <https://tygs.dsmz.de>), applying the recommended formula 2 [28].

Protein modeling

For structural modeling, every enzyme was submitted to the AlphaFold3 web service and processed under its factory settings [29]. Visualization of the resulting three-dimensional models was handled in PyMOL.

Transcriptomic analysis

Agarivorans sp. D1326 was inoculated into six flasks each containing 100 mL of 2216 liquid medium. The cultures were incubated at 28°C with shaking at 220 rpm for 6 hours to reach the logarithmic growth phase. Bacterial cells were then harvested by centrifugation at 4000 rpm for 5 minutes. The cell pellets

from three flasks served as the control group (C group). The cell pellets collected from the remaining three flasks were resuspended in 100 mL of 2216 medium supplemented with 0.25% (w/v) agarose and incubated for an additional 4 hours under the same conditions. Subsequently, cells were harvested by centrifugation to constitute the agarose induction group (I group). The harvested C group and I group samples were sent to Majorbio Biomedical Technology Co., Ltd. (Shanghai, China) for RNA sequencing (RNA-seq). Detailed methods are provided in the supplementary information.

Proteomic analysis

Extracellular protein samples were prepared from the culture broth of *Agarivorans* sp. D1326 both before and after 4 hours of agarose induction. Following centrifugation to collect the bacterial cells, the supernatant was lyophilized to obtain the extracellular protein fraction. For intracellular proteins, the cell pellets collected before and after induction were resuspended in SDS lysis buffer (Beyotime, China) and disrupted by ultrasonication. The lysate was then centrifuged, and the resulting supernatant was lyophilized to obtain the intracellular protein samples. Protein digestion was performed using filter-aided sample preparation (FASP). Mass spectrometry was performed on an Orbitrap Fusion Lumos (Thermo Fisher, USA). Data-independent acquisition (DIA) data were processed using Spectronaut 18 (Biognosys, Switzerland) with direct DIA mode. Detailed methods are provided in the supplementary information.

Results

Phenotypic characterizations of strain D1326

Seventy-one strains were isolated and purified from marine red algae sample. A strain designated D1326^T exhibited highly agarolytic activities, showing a clear hydrolysis zone on MA after cultivation at 28 °C for 96 h (Fig. S1). This strain has been preserved at the China Center for Type Culture Collection (CCTCC), with preservation number: M20242935. Colonies of strain D1326^T were circular, smooth, opaque, and white with a diameter of approximately 1–2 mm when cultured on MA medium at 28°C for 48 h. Growth of strain D1326^T occurred at 8–5°C, with an optimal temperature of 28°C. Growth occurs in appropriately modified synthetic marine ZoBell broth with 1.08–6.0% (w/v) NaCl (optimum 3.0%). The pH range for growth is 5.08 – 10.0 (optimum pH 7.0) (Table S2). According to the results of API 20E, strain D1326^T could produce acid from glucose and mannose, but not from inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin, and arabinose. Urea and gelatin could not be hydrolyzed by strain D1326^T. By comparing the physiological and biochemical characteristics with other agar-utilizing marine strains, strain D1326^T was found to be highly similar to them (Table S2).

Phylogenetic analysis based on the 16S rRNA

The nearly complete 16S rRNA gene sequence (1506 nt) of strain D1326^T was deposited in GenBank and assigned the accession number PX780718. Phylogenetic analysis based on the maximum-likelihood

method revealed that strain D1326^T formed a distinct cluster with the closely related type strains *Agarivorans litoreus* GJSW-6^T (99.1%) and *A. albus* MKT 106^T (98.6% identity), placing it securely within the radiation of the genus *Agarivorans* (Fig. S2).

Phylogenomic analysis

The draft genome of strain D1326^T comprised 28 scaffolds, totaling 4.69 Mb, and was predicted to encode 4,185 proteins (Table S3, Fig. S3). The completeness is 99.32% and the contamination is 1.35% evaluated using CheckM implemented in NCBI (Table S3). The DNA G + C content of strain D1326^T was 44.5%, closely to other strains in the genus *Agarivorans* (Table S3). In the phylogenomic tree, strain D1326^T formed a distinct clade with *A. albus* MKT 106^T and *A. aestuarii* hyd662^T, showing a closer affiliation to *A. albus* MKT 106^T. This clade, in turn, grouped with *A. litoreus* GJSW-6^T. Accordingly, strain D1326^T fell within the genus *Agarivorans* cluster (Fig. 1). This phylogenomic placement further supports the assignment of strain D1326^T to the genus *Agarivorans*. The phylogenomic tree inference was strongly supported by overall genome relatedness indices. Strain D1326^T exhibited the highest ANI (96.86%) and DDH (75.10%) values with *A. albus* MKT 106^T (Table S4). Thus, strain D1326^T belongs to *A. albus*.

Genome-based profiling of agarolytic enzymes

Genomic analysis of *Agarivorans* sp. D1326 identified 182 Carbohydrate-Active enZYmes (CAZy) modules (Fig. 2a), among which 97 genes (53.3%) encode glycoside hydrolases (GH). Genomic annotation of *Agarivorans* sp. D1326 revealed a cluster encoding five putative GH16 agarases, four GH50 agarases, two GH86 agarases, one GH118 agarase, one GH117 neoagarobiose hydrolase, two AHG dehydrogenases, one 3,6-L-anhydrogalactonate (AHGA) cycloisomerase, one L-galactonate-5-dehydrogenase, two α -galactosidases, one β -galactosidase, one β -carrageenase, and one β -porphyranase (Fig. 2, Table S5).

In *Agarivorans* sp. D1326, all five GH16 agarases possess this catalytic domain in conjunction with carbohydrate-binding modules (CBMs) (Fig. S4, Table S5). Detailed structural analysis reveals: Aga1448 (75.22% sequence identity to *Vibrio* sp. AgaV, UniProt ID: A1E2B6); Aga2214 (50.76% identity to *Z. galactanivorans* AgaD catalytic domain, PDB ID: 4ASM); Aga2205 (46% identity to *Z. galactanivorans* β -agarase B, PDB ID: 1O4Z); Aga15 (56.63% identity to *Z. galactanivorans* AgaC, PDB ID: 6HY3); Aga2279 (29.72% identity to *Wenyngzhuangia fucanilytica* κ -carrageenase Cgbk16A catalytic domain, PDB ID: 9IJC). All enzymes contain N-terminal signal peptides, with extracellular localization confirmed for four enzymes, whereas Aga2205 demonstrates ambiguous cellular localization (Table S5).

Crystallographically resolved GH50 agarases include AgWH50C (PDB ID: 5Z6P) from *A. gilvus* WH0801, Aga50D (PDB ID: 4BQ2) from *S. degradans* 2–40, and BpGH50 (PDB ID: 5T3B) from *B. plebeius* DSM 17135. The canonical structure of GH50 family agarases features an (α/β)₈ TIM barrel catalytic domain core supplemented by carbohydrate-binding modules (CBMs). Homology modeling of four GH50

agarases from *Agarivorans* sp. D1326 reveals highly conserved catalytic domains (Fig. S5) with significant structural similarity to AgWH50C, while exhibiting CBM divergence: Aga1474 contains one CBM whereas the others possess two CBMs. Notably, Aga1474 lacks an N-terminal signal peptide, contrasting with the signal peptide-bearing Aga1459, Aga1466, and Aga1467. Subcellular localization predictions indicate distinct compartments: outer membrane (Aga1459), periplasm (Aga1466), extracellular space (Aga1467), and cytoplasm (Aga1474) (Table S5). In this study, phylogenetic analysis of reported GH50 agarases and four GH50 members from *Agarivorans* sp. D1326 (Fig. S6) revealed close evolutionary relationships: Aga1459 is phylogenetically affiliated with VejGH50A from *Vibrio* sp. EJY3 (57.9% sequence identity, Fig. S7); Aga1466 clusters with AgWH50B from *A. gilvus* WH0801 (72.16% identity, Fig. S8); Aga1474 shows proximity to AgWH50C (65.53% identity, Fig. S19); and Aga1467 exhibits near-identity (99.7%, Fig. S10) with AgaA11 from *Agarivorans* sp. JAMB-A11.

Genomic analysis of *Agarivorans* sp. D1326 in this study identified two GH86 agarases, designated Aga1002 and Aga1341 (Table S5). Homology modeling revealed that their catalytic domains exhibit high structural similarity to each other and strong conservation with the catalytic domain of Aga86A_Wa from *W. aestuarii* (Fig. S11). The catalytic domain of Aga1002 in this study shares the highest sequence identity (34%) with Aga86A_Wa (PDB ID: 8H97) based on SWISS-MODEL modeling. Amino acid sequence alignment predicted the corresponding putative catalytic residues in Aga1002 to be E786 and E951 (Fig. S12). Furthermore, three-dimensional structural comparison (Fig. S13) indicated that Aga1002 possesses three N-terminal CBMs followed by a catalytic domain, which exhibits high structural similarity to Aga86A_Wa, particularly within the catalytic center, and presumably hydrolyze agarose to yield NA2.

Genomic analysis of *Agarivorans* sp. D1326 identified a novel GH118 agarase, Aga2820, which contains an N-terminal signal peptide and is predicted to localize extracellularly (Table S5). Structural characterization of GH118 enzymes remains unexplored; however, AlphaFold3 modeling of Aga2820 and other GH118 β -agarases revealed a distinctive β -helix fold (Fig. S14). Furthermore, the role of GH118 agarases in agarose catabolism is currently unelucidated.

Following the degradation of agarose into NA2 by β -agarase, it can be further hydrolyzed into galactose and L-AHG under the catalysis of NA2 hydrolase (NABH) [18]. The enzyme NABH1462 of *Agarivorans* sp. D1326, exhibits high sequence identity with these two enzymes: 82% identity to VejGH117 from *Vibrio* sp. EJY3 [16] and 80% identity to AgaWH117 from *A. gilvus* WH0801[30] (Fig. S15). Bioinformatic analysis further confirmed that it belongs to GH117 family, consistent with the family classification of the reported NA2 hydrolases (Table S5).

L-AHG is further converted to 2-keto-3-deoxy-galactonate (KDGal) through sequential catalysis by AHG dehydrogenase (AHGD) and AHGA cycloisomerase (AHGC) [31, 32]. Genomic analysis in this study confirmed the presence of these enzymes: AHGD1449 and AHGD2206 as AHG dehydrogenases, and AHGC1452 as an AHGA cycloisomerase (Table S5). While the crystal structure of AHGD from *Vibrio variabilis* JCM 19239 (VvAHGD) has been resolved [33], homology modeling of AHGD1449 and

AHGD2206 revealed high structural congruence with VvAHGD (Fig. S16). Comparative sequence analysis revealed that AHGD1449 shares 47.48% identity with VvAHGD, while AHGD2206 exhibits significantly higher identity (67.43%). The crystal structure of the AHGC enzyme from the marine bacterium *Vibrio* sp. strain EJY3 has been determined [34]. The homology modeling model of AHGC1452 exhibits high structural consistency with this resolved structure (Fig. S17). Sequence alignment analysis revealed a sequence identity of 78.45% between the two.

Transcriptomic analysis

After transferring *Agarivorans* sp. D1326 from 2216 medium with glucose as the carbon source to an agarose-based medium (Fig. 3a), the cells rapidly adapt to the new environment and continue to grow quickly. Compared to cells originally cultured in 2216 medium, they ultimately achieve a higher final biomass. This also indicates that this strain possesses the ability to rapidly utilize agarose. Comparative transcriptomics of bacterial cells before and after agarose induction revealed upregulation of 746 genes and downregulation of 611 genes (Fig. 3b). Based on KEGG enrichment analysis of the top 20 relevant pathways, we observed significant changes in the expression of genes associated with transporter activity after switching the carbon source from glucose to agarose (Fig. S18). This suggests that a large number of enzymes are likely secreted into the culture medium, while substantial amounts of metabolic products are transported into the cells (Fig. 3c). Furthermore, expression levels of beta-agarase-related genes changed markedly.

As predicted by prior analyses, agarose induction markedly upregulated multiple agarase-encoding genes across distinct GH families in *Agarivorans* sp. D1326 (Fig. 3f). The most pronounced induction was observed for *gene1002* (292.64-fold), encoding the GH86 agarase Aga1002. Significant upregulation also occurred for the GH16 agarase gene *gene1448* (> 100-fold) and the GH118 agarase gene *gene2820* (45.54-fold), encoding Aga2820. Multiple GH50 agarases genes exhibited substantial induction: *gene1459* (83.10-fold), *gene1466* (31.51-fold), *gene1467* (35.28-fold), and *gene1474* (19.17-fold). These findings collectively demonstrate the concerted action of GH16, GH50, GH86, and GH118 family agarases in agarose utilization. Concomitantly, the transcription level of *gene1462* encoding a GH117 family NA2 hydrolase was significantly upregulated by 10.48-fold (Fig. 3f). This indicates that during agarose utilization by *Agarivorans* sp. D1326, agarose is ultimately depolymerized into monomeric sugars. As expected, genes encoding enzymes for subsequent L-AHG catabolism were robustly induced: *gene1449* (encoding AHG dehydrogenase; 171.09-fold) and *gene1457* (encoding L-galactonate-5-dehydrogenase; 213.28-fold) exhibited the most significant responses (Fig. 3f). This divergent transcriptional regulation suggests preferential activation of downstream L-AHG metabolic pathways during agarose utilization in *Agarivorans* sp. D1326.

As predicted, substitution of glucose with agarose as the carbon source induces extracellular secretion of these agarases to initiate enzymatic hydrolysis of agarose. Transcriptome profiling demonstrated significant upregulation of Twin-Arginine Translocation (Tat) protein export system components: *gene0006* (protein translocase TatB; 99.38-fold increase) and *gene0007* (protein translocase TatA; 88.1-fold increase). Conversely, General Secretory (Sec) protein export system genes exhibited marked

downregulation (*gene2354* and *gene4553*), while *gene3611* expression decreased to 0.66-fold of pre-induction levels despite lacking statistical significance (Fig. 3e, Table S6).

Transcriptomic analysis revealed that ATP-binding cassette (ABC) transporter-related genes (e.g., *gene2840*, *gene2848*, and *gene2841*, each > 250-fold) and two Major Facilitator Superfamily (MFS) transporter genes (*gene1451*: 245.59-fold; *gene1464*: 38.58-fold) were upregulated in *Agarivorans* sp. D1326, whereas two glucose-specific phosphoenolpyruvate-dependent phosphotransferase system (PTS) transporter genes (*gene1106* and *gene3664*) were significantly downregulated (Fig. 3d, Table S7).

Proteomic analysis

Based on prior genomic and transcriptomic analyses, agarose induction transcriptionally activated agarose-degrading enzymes in *Agarivorans* sp. D1326. SDS-PAGE of intracellular proteins revealed significant composition and abundance changes after induction (Fig. 4a). To further investigate key enzyme systems governing agarose utilization, including subcellular localization and the role of the poorly characterized GH118 agarase Aga2820, we performed comparative proteomics on culture supernatants (extracellular) and cell lysates (intracellular) before and after agarose induction.

Our results demonstrate substantial alterations in protein expression profiles within both the culture supernatant (Fig. 4b) and intracellular compartments (Fig. 4c). Notably, significant expression changes were observed in agarose-degrading enzymes (Fig. 4d), representing our primary focus. Proteomics revealed substantial induction of GH16 β -agarases upon agarose exposure, with distinct subcellular patterns. Aga2799 increased 299.69-fold (extracellular) and 5.09-fold (intracellular). Aga1448 was upregulated 25.84-fold in the supernatant but downregulated intracellularly (0.71-fold). Aga2205 and Aga2214 showed parallel extracellular/intracellular induction: 52.43/69.59-fold and 20.33/4.95-fold, respectively.

Concurrent with GH16 induction, four GH50 family agarases (Aga1459, Aga1466, Aga1467, and Aga1474) exhibited significant transcriptional upregulation in *Agarivorans* sp. D1326. Notably, Aga1467 demonstrated the most pronounced induction with 103.49-fold and 80.29-fold increases in extracellular and intracellular abundance, respectively. Unlike the compartmentalized regulation observed in GH16 enzymes, all characterized GH50 isoforms displayed comparable induction magnitudes between extracellular and intracellular environments (< 2.5-fold differential), suggesting distinct subcellular trafficking mechanisms. This striking contrast to the extracellular-dominant induction pattern of GH16 agarases implies functional specialization within the agarolytic enzyme repertoire, where GH50 enzymes may operate through coordinated intra- and extracellular action rather than polarized secretion.

Concurrently, two additional agarases exhibited extraordinary induction: GH86 family member Aga1002 and GH118 family enzyme Aga2820. These enzymes demonstrated 443.97-fold and 303.72-fold extracellular upregulation, respectively, contrasted with modest intracellular increases of 6.06-fold and 16.92-fold (Fig. 4d). This compartmentalized induction pattern-mirroring that of GH16 agarases-implies efficient secretion for extracellular agarose depolymerization.

GH117 NABH is responsible for hydrolyzing NA2 to AHG and galactose [18]. Proteomic analysis demonstrated that upon agarose induction, the expression levels of GH117 NABH1462 was significantly upregulated by 36.96-fold intracellularly and 19.97-fold in the culture supernatant. AHG can be firstly converted to AHGA by AHG dehydrogenase (AHGD) [33], which is subsequently transformed into KDGal under the catalysis of AHGA cycloisomerase (AHGC).. In this study, we observed that both type enzymes in *Agarivorans* sp. D1326 exhibited significantly upregulated expression upon agarose induction: AHGD1449 and AHGD2206 showed 46.31-fold and 21.53-fold upregulation intracellularly, and 24.30-fold and 22.63-fold upregulation in the culture supernatant, respectively; AHGC1452 demonstrated 5.68-fold intracellular and 20.34-fold extracellular upregulation.

In this study, we also observed that the expression of the L-galactonate 5-dehydrogenase GOD1457 encoded by *gene1457* was significantly upregulated upon agarose induction (26.42-fold intracellularly and 30.16-fold in the supernatant). This enzyme catalyzes the conversion of L-galactonate to D-tagaturonate, which is ultimately cleaved into pyruvate and D-glyceraldehyde for entry into central carbon metabolism [35]. However, neither L-galactonate synthase nor D-tagaturonate lyase was identified in the genome, indicating this pathway remains incompletely resolved. Notably, α/β -galactosidases (Agl1497, Agl552, Bgl553) and the porphyranase Por2270 (encoded by *gene2270*) showed no significant upregulation after induction, highlighting the strain's specific adaptation to agarose degradation.

Discussion

Our group has long been dedicated to the mining and engineering of agarase-producing strains and agarase resources [36] [37]. In this study, we isolated an agarolytic strain from a marine algal sample collected along the coastline of the Yellow Sea. Physiological and biochemical characterization revealed that strain D1326^T shares high similarity with members of the genus *Agarivorans*. Both phylogenetic and phylogenomic analyses support the classification of strain D1326^T as a novel representative of this genus. The highest ANI (97.09%) and DDH (75.10%) values further confirmed that strain D1326^T belongs to the same species as *Agarivorans* albus MKT 106^T [38].

The degradation and utilization of agar constitute a complex process that requires the synergistic action of multiple agarases from different families. Genomes of all known agar-degrading bacteria have been found to harbor agarose-degrading enzymes from various families, with some families represented by more than one coding gene. For example, the genome of *Paraglaciecola hydrolytica* S66^T contains six GH16 β -agarases, four GH50 β -agarases, two GH86 β -agarases, one GH117 NA2 hydrolase, as well as multiple enzymes involved in L-AHG metabolism [39, 40]. In contrast, *Saccharophagus degradans* 2-40^T possesses one GH16 β -agarase, two GH86 β -agarases, and two GH50 β -agarases [17]. In this study, through genomic analysis of *Agarivorans* sp. D1326, we also found that this strain harbors multiple agar-degrading enzymes from different families, including four GH16 β -agarases, four GH50 β -agarases, two GH86 β -agarases, one GH118 β -agarase, one GH117 NA2 hydrolase, and several enzymes involved in L-AHG metabolism. To date, this study represents the first genomic analysis of an agar-degrading

bacterium that contains a GH118 family agarase. Furthermore, through transcriptomic and proteomic approaches, we have elucidated the role of the GH118 family agarase in agar metabolism.

Genomic analysis of *Agarivorans* sp. D1326 revealed multiple agarases from different GH families (Fig. 1, Table S5). However, key questions remain: whether GH118 agarase participates in agar degradation and if its activity is intra- or extracellular, and the roles of other agarases. Prior research on *Vibrio fluvialis* A8 employed multi-omics method to characterize its agarose degradation pathway [41]. In this study, we performed transcriptomic and proteomic researches to address the key role of GH118 agarase in the agarose utilization network.

When the carbon source in the culture medium of *Agarivorans* sp. D1326 was switched from glucose to agarose, transcriptomic and proteomic analyses revealed that the transcriptional and translational levels of a series of agar-degrading enzyme genes were rapidly upregulated. Simultaneously, large amounts of agarases were secreted into the extracellular environment, contributing to agarose degradation. Under the condition with agarose as the sole carbon source, bacterial biomass increased rapidly, indicating that the degradation products of agarose were transported into cells and participated in cellular metabolism and bacterial proliferation (Fig. 3, Fig. 4).

Bacteria utilize two principal pathways for protein translocation across the cytoplasmic membrane: the Sec Pathway and the Tat pathway [42]. When agarose replaced glucose as the carbon source, *Agarivorans* sp. D1326 upregulated the Tat protein export system and downregulated selected Sec system components (Fig. 3e, Table S6). Our transcriptomic profiling of *Agarivorans* sp. D1326 provides the first direct evidence that agarose metabolism triggers coordinated reprogramming of the protein export machinery. The functional relevance of this native mechanism is further supported by heterologous studies, in which engineering the Tat pathway in *Corynebacterium glutamicum* enabled efficient secretion of *Streptomyces coelicolor* β -agarase (DagA) [43].

ABC transporters actively pump diverse substrates across cellular membranes using ATP hydrolysis [44]; Major Facilitator MFS is the largest secondary active transporter family, moving diverse substrates [45]; and glucose-specific PTS transport with substrate phosphorylation [46]. Agarose induced upregulation of ABC (> 250-fold) and MFS transporters, and downregulation of PTS genes (Fig. 3d, Table S6). This suggests ABC transporters dominate uptake of agarose-derived sugars. The concurrent MFS upregulation implies cooperative ABC-MFS transport. PTS downregulation reflects carbon-source-dependent reprogramming, consistent with the shift from glucose to agarose.

In this study, transcriptomic and proteomic results indicate that genes involved agarose degradation process with elevated transcriptional levels show correspondingly increased translational levels, remaining largely consistent (Fig. 3, 4). The top three most upregulated enzymes are GH86 β -agarase Aga1002, GH118 β -agarase Aga2820, and GH16 β -agarase Aga2799. All three enzymes are highly expressed in the culture supernatant while showing significantly lower intracellular expression (Fig. 4), indicating their predominant secretion after intracellular synthesis, consistent with their predicted localization (Table S5). Given that GH16 family enzymes directly hydrolyze agarose into the predominant

product NA4 and serve as the primary depolymerization system in agarolytic microbes [18], our findings collectively indicate that multiple GH16 agarases encoded by distinct genes mediate agarose catabolism in *Agarivorans* sp. D1326. Extracellular degradation appears to be the principal mechanism, as supported by the predominant localization of these enzymes and their substantial induction in the culture supernatant. Structural analysis reveals that GH118 agarases possess more open substrate-binding pockets than their GH16 counterparts, which is consistent with prior biochemical evidence confirming their direct activity on agarose [10, 11, 13, 18]. Furthermore, the observed extracellular localization of Aga2820, together with its confirmed substrate specificity, establishes this poorly characterized GH118 family member as a quantitatively predominant agarase within the agarolytic system of *Agarivorans* sp. D1326. All three GH β -agarase families (GH86, GH118, and GH16) directly utilize agarose as a substrate but yield distinct hydrolysis products: NA2 from GH86, NA8/NA10 from GH118, and NA4 from GH16 [11, 18, 47]. Therefore, we propose that the initial step of agarose degradation by *Agarivorans* sp. D1326 proceeds through three major pathways mediated by these enzymes (marked with red, green, and blue lines in Fig. 5). Additionally, other upregulated GH16 agarases detected in the culture fluid (Aga2205, Aga2214, and Aga1448) likely contribute to partial agarose degradation.

Unlike Aga1002, Aga2820, and Aga2799, GH50 agarases exhibit minimal differential expression between intracellular and extracellular compartments (Fig. 4). Currently documented GH50 agarases are primarily classified into two functional categories: (1) endolytic enzymes that depolymerize agarose into NA4 as the dominant oligosaccharide [48], and (2) exolytic enzymes yielding NA2 as the end product [49]. Based on phylogenetic proximity, we propose that Aga1466 functions as an endolytic enzyme producing NA4, while the other three (Aga1459, Aga1467, Aga1474) are exolytic enzymes generating NA2 (Fig. S12). Therefore, we propose supplementary extracellular agarose degradation pathways mediated by GH50 agarases: 1) Aga1467/Aga1459/Aga1474-catalyzed conversion to NA2, and 2) Aga1466-catalyzed conversion to NA4 (Fig. 5).

Agarases belonging to the GH86, GH16, and GH50 families are capable of utilizing not only agarose but also short-chain agarooligosaccharides as substrates, catalyzing their degradation into lower molecular weight agarooligosaccharides [18]. Consequently, the GH118 agarase Aga2820 hydrolysis products (such as NA8) can be further degraded to NA2 by agarases such as Aga1002 (GH86) or Aga1467/Aga1459/Aga1474 (GH50), or to NA4 by agarases such as Aga2799/Aga2205/Aga2214/Aga1448 (GH16) or Aga1466 (GH50). In currently reported agarose degradation pathways, NA4 and NA2 can be transported into the cytoplasm via transporter proteins located on the cell membrane, such as ABC transporters. This study also identified, through transcriptomic analysis, the significant upregulation of multiple genes encoding ABC transporters (Fig. 3). Furthermore, the intracellular expression levels of the GH50 family agarases Aga1467, Aga1459, and Aga1474 were also markedly increased. This suggests that these intracellular agarases may further degrade a portion of the NA4 transported into the cell down to NA2. Therefore, the conversion of NA4 to NA2 is likely to occur both intra- and extracellularly (Fig. 5).

GH117 NABH is responsible for hydrolyzing NA2 to AHG and galactose [18]. Current studies consistently indicate that intracellular NA2 is hydrolyzed by NA2 hydrolase (NABH) into galactose and AHG. L-AHG is further converted to 2-keto-3-deoxy-galactonate (KDGal) through sequential catalysis by AHG dehydrogenase (AHGD) and AHGA cycloisomerase (AHGC), enabling its entry into the Entner-Doudoroff pathway to integrate into central carbon metabolism alongside galactose [31, 32, 39, 40]. However, this study observed significant upregulation of the NABH enzyme NABH1462, detected in both supernatant (19.97-fold increase) and intracellular fractions (36.96-fold increase). Notably, although NABH1462 lacks a signal peptide and is predicted to localize cytoplasmically (Table S5), its extracellular presence raises the possibility of alternative translocation mechanisms. This warrants further investigation, particularly given precedents in other bacteria. For instance, in Gram-negative *Escherichia coli*, hemolysin A is secreted extracellularly via Type 1 secretion systems (T1SSs) independent of N-terminal signal peptides, utilizing three ABC transporters [50]. Similarly, AHG-degrading enzymes AHGD1449/AHGD2206 and AHGC1452 in this study also lack signal peptides yet exhibit markedly elevated expression in supernatant (Fig. 4; Table S5). Based on these experimental findings and existing literature, we propose that AHG degradation in *Agarivorans* sp. D1326 might occur both extracellularly and intracellularly during agar utilization.

Conclusion

This study isolated the marine bacterium *Agarivorans* sp. D1326, which harbors agarase genes from GH16, GH50, GH86, and the rare GH118 families. Multi-omics revealed the complete agarose utilization pathway: upon induction, multiple agarases were synergistically upregulated (transcriptionally and translationally) and secreted predominantly via the Tat pathway. We report the first demonstration of a GH118 agarase's significant upregulation in a native pathway. Data also show coordinated oligosaccharide uptake by ABC and MFS transporters, Tat-mediated export of folded agarases, and GH117-catalyzed hydrolysis of NA2 to monomers. This work underscores GH118's crucial role in the native degradation network, expands understanding of this family, and provides enzyme resources and a theoretical foundation for green production of agar oligosaccharides.

Declarations

Supplementary information

The online version contains supplementary material available at

Declarations

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding

This work was supported by the Natural Science Foundation of Henan Province (grant number 252300423147); the Joint Funds of the Science and Technology Research and Development Plan of Henan Province (grant number 252103810361); the University-Enterprise Collaborative Innovation Project of Henan Province (grant number 26AXQXT056); and the National Program of College Student Innovation and Entrepreneurship Training (grant number 202510472009).

Author Contribution

Tao Li: Writing – original draft, Conceptualization, Funding acquisition. Cong Liu: Data curation, Formal analysis, Visualization. Chengwei Wu: Investigation, Validation. Boyu Zhang: Investigation, Formal analysis. Ziwei Wang: Investigation. Yueyue Zhao: Data curation. Wenbin Gu: Data curation. Zilin Liu: Data curation. Fanling Meng: Visualization. Jingjing Shangguan: Data curation. Wenbo Zhang: Methodology. Xueyi Zhu: Resources. Yan Wang: Writing – review & editing, Funding acquisition, Supervision.

Data availability

The proteomics data generated in this study are available in the PRIDE database under accession number PXD072825. The genomic and transcriptomic data have been deposited in the SRA database, with the accession numbers JBTWEN0000000000 and PRJNA1404874, respectively.

References

1. Martin M, Portetelle D, Michel G, Vandenbol M (2014) Microorganisms living on macroalgae: diversity, interactions, and biotechnological applications. *Appl Microbiol Biotechnol* 98:2917–2935. <https://doi.org/10.1007/s00253-014-5557-2>
2. Pathiraja D, Christiansen L, Park B, Schultz-Johansen M, Bang G, Stougaard P, Choi I-G (2021) A Novel Auxiliary Agarolytic Pathway Expands Metabolic Versatility in the Agar-Degrading Marine Bacterium *Colwellia echini* A3T. *Appl Environ Microbiol* 87:e00230–e00221. <https://doi.org/10.1128/AEM.00230-21>
3. Kumar BR, Mathimani T, Sudhakar MP, Rajendran K, Nizami AS, Brindhadevi K, Pugazhendhi A (2021) A state of the art review on the cultivation of algae for energy and other valuable products: Application, challenges, and opportunities. *Renew Sustainable Energy Rev* 138:110649. <https://doi.org/10.1016/j.rser.2020.110649>
4. Popper ZA, Michel G, Hervé C, Domozych DS, Willats WGT, Tuohy MG, Kloareg B, Stengel DB (2011) Evolution and diversity of plant cell walls: from algae to flowering plants. *Annu Rev Plant Biol* 62:567–590. <https://doi.org/10.1146/annurev-arplant-042110-103809>

5. Li Z, Hu Q, Chen J, Liu X, Sang Y, Wang J, Zheng M, Ni H, Li Q, Jiang Z, Zhu Y (2022) Optimized strategy for simultaneous recovering bioactive oligosaccharides and reusable perlite from agar industrial waste residues. *J Clean Prod* 378:134631. <https://doi.org/10.1016/j.jclepro.2022.134631>
6. Usov AI (1992) Sulfated polysaccharides of the red seaweeds. *Food Hydrocolloids* 6:9–23. [https://doi.org/10.1016/S0268-005X\(09\)80055-6](https://doi.org/10.1016/S0268-005X(09)80055-6)
7. Zheng Y, Li Y, Yang Y, Zhang Y, Wang D, Wang P, Wong ACY, Hsieh YSY, Wang D (2022) Recent advances in bioutilization of marine macroalgae carbohydrates: Degradation, metabolism, and fermentation. *J Agric Food Chem* 70:1438–1453. <https://doi.org/10.1021/acs.jafc.1c07267>
8. Jagtap AS, Manohar CS (2021) Overview on microbial enzymatic production of algal oligosaccharides for nutraceutical applications. *Mar Biotechnol* 23:159–176. <https://doi.org/10.1007/s10126-021-10027-6>
9. Jahromi ST, Barzkar N (2018) Future direction in marine bacterial agarases for industrial applications. *Appl Microbiol Biotechnol* 102:6847–6863. <https://doi.org/10.1007/s00253-018-9156-5>
10. Wu YR, Zhou ZR, Zhao M, Lin B, Zhong M, Hu Z (2017) Molecular characterization of the thermostability and carbohydrate-binding module from a newly identified GH118 family agarase, AgaXa. *Process Biochem* 52:192–199. <https://doi.org/10.1016/j.procbio.2016.10.021>
11. Ma C, Lu X, Shi C, Li J, Gu Y, Ma Y, Chu Y, Han F, Gong Q, Yu W (2007) Molecular cloning and characterization of a novel beta-agarase, AgaB, from marine *Pseudoalteromonas* sp. CY24. *J Biol Chem* 282:3747–3754. <https://doi.org/10.1074/jbc.M607888200>
12. Dong J, Hashikawa S, Konishi T, Tamaru Y, Araki T (2006) Cloning of the novel gene encoding β -agarase C from a marine bacterium, *Vibrio* sp. strain PO-303, and characterization of the gene product. *Appl Environ Microbiol* 72:6399–6401. <https://doi.org/10.1128/AEM.00935-06>
13. Lee DG (2012) Cloning, expression, and characterization of a glycoside hydrolase family 118 β -agarase from *Agarivorans* sp. JA-1. *J Microbiol Biotechnol* 22:1692–1697. <https://doi.org/10.4014/jmb.1209.09033>
14. Chi WJ, Chang YK, Hong SK (2012) Agar degradation by microorganisms and agar-degrading enzymes. *Appl Microbiol Biotechnol* 94:917–930. <https://doi.org/10.1007/s00253-012-4023-2>
15. Hehemann JH, Correc G, Thomas F, Bernard T, Barbeyron T, Jam M, Helbert W, Michel G, Czejek M (2012) Biochemical and structural characterization of the complex agarolytic enzyme system from the marine bacterium *Zobellia galactanivorans*. *J Biol Chem* 287:30571–30584. <https://doi.org/10.1074/jbc.M112.377184>
16. Yu S, Yun EJ, Kim DH, Park SY, Kim KH (2020) Dual agarolytic pathways in a marine bacterium, *Vibrio* sp. strain EJY3: Molecular and enzymatic verification. *Appl Environ Microbiol* 86:e02724–e02719. <https://doi.org/10.1128/AEM.02724-19>
17. Ekborg NA, Taylor LE, Longmire AG, Henrissat B, Weiner RM, Hutcheson SW (2006) Genomic and proteomic analyses of the agarolytic system expressed by *Saccharophagus degradans* 2–40. *Appl Environ Microbiol* 72:3396–3405. <https://doi.org/10.1128/AEM.72.5.3396-3405.2006>

18. Jiang C, Liu Z, Cheng D, Mao X (2020) Agarose degradation for utilization: Enzymes, pathways, metabolic engineering methods and products. *Biotechnol Adv* 45:107641. <https://doi.org/10.1016/j.biotechadv.2020.107641>
19. Liang Y, Ma X, Zhang L, Li F, Liu Z, Mao X (2017) Biochemical characterization and substrate degradation mode of a novel exotype β -agarase from *Agarivorans gilvus* WH0801. *J Agric Food Chem* 65:7982–7988. <https://doi.org/10.1021/acs.jafc.7b01533>
20. Lee HK, Jang WY, Kim YH (2023) Extracellular production of a thermostable *Cellvibrio* endolytic β -agarase in *Escherichia coli* for agarose liquefaction. *AMB Express* 13:42. <https://doi.org/10.1186/s13568-023-01551-w>
21. Pluvinaige B, Grondin JM, Amundsen C, Klassen L, Moote PE, Xiao Y, Thomas D, Pudlo NA, Anele A, Martens EC, Inglis GD, Uwiera RER, Boraston AB, Abbott DW (2018) Molecular basis of an agarose metabolic pathway acquired by a human intestinal symbiont. *Nat Commun* 9:1043. <https://doi.org/10.1038/s41467-018-03366-x>
22. Beveridge TJ, Lawrence JR, Murray RGE (2007) Sampling and staining for light microscopy. In: *Methods for General and Molecular Microbiology*. pp 19–33
23. Yoon SH, Ha SM, Kwon S, Lim J, Kim Y, Seo H, Chun J (2017) Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. *Int J Syst Evol Microbiol* 67:1613–1617. <https://doi.org/10.1099/ijsem.0.001755>
24. Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 32:1792–1797. <https://doi.org/10.1093/nar/gkh340>
25. Tamura K, Stecher G, Kumar S (2021) MEGA11: Molecular evolutionary genetics analysis version 11. *Mol Biol Evol* 38:3022–3027. <https://doi.org/10.1093/molbev/msab120>
26. Luo R, Liu B, Xie Y, Li Z, Huang W, Yuan J, He G, Chen Y, Pan Q, Liu Y, Tang J, Wu G, Zhang H, Shi Y, Liu Y, Yu C, Wang B, Lu Y, Han C, Cheung DW, Yiu S, Peng S, Xiaoqian Z, Liu G, Liao X, Li Y, Yang H, Wang J, Lam TW, Wang J (2012) SOAPdenovo2: an empirically improved memory-efficient short-read de novo assembler. *Gigascience* 1:2047-217X-1–18. <https://doi.org/10.1186/2047-217X-1-18>
27. Chaumeil P-A, Mussig AJ, Hugenholtz P, Parks DH (2022) GTDB-tk v2: memory friendly classification with the genome taxonomy database. *Bioinformatics* 38:5315–5316
28. Meier-Kolthoff JP, Carbasse JS, Peinado-Olarte RL, Göker M (2022) TYGS and LPSN: a database tandem for fast and reliable genome-based classification and nomenclature of prokaryotes. *Nucleic Acids Res* 50:D801–D807. <https://doi.org/10.1093/nar/gkab902>
29. Abramson J, Adler J, Dunger J, Evans R, Green T, Pritzel A, Ronneberger O, Willmore L, Ballard AJ, Bambrick J, Bodenstein SW, Evans DA, Hung CC, O'Neill M, Reiman D, Tunyasuvunakool K, Wu Z, Žemgulytė A, Arvaniti E, Beattie C, Bertolli O, Bridgland A, Cherepanov A, Congreve M, Cowen-Rivers AI, Cowie A, Figurnov M, Fuchs FB, Gladman H, Jain R, Khan YA, Low CMR, Perlin K, Potapenko A, Savy P, Singh S, Stecula A, Thillaisundaram A, Tong C, Yakneen S, Zhong ED, Zielinski M, Židek A, Bapst V, Kohli P, Jaderberg M, Hassabis D, Jumper JM (2024) Accurate structure prediction of

- biomolecular interactions with AlphaFold 3. *Nature* 630:493–500. <https://doi.org/10.1038/s41586-024-07487-w>
30. Liu N, Yang M, Mao X, Mu B, Wei D (2016) Molecular cloning and expression of a new α -neoagarobiose hydrolase from *Agarivorans gilvus* WH0801 and enzymatic production of 3,6-anhydro-L-galactose. *Biotechnol Appl Biochem* 63:230–237. <https://doi.org/10.1002/bab.1363>
 31. Lee SB, Cho SJ, Kim JA, Lee SY, Kim SM, Lim HS (2014) Metabolic pathway of 3,6-anhydro-L-galactose in agar-degrading microorganisms. *Biotechnol Bioprocess Eng* 19:866–878. <https://doi.org/10.1007/s12257-014-0622-3>
 32. Yun EJ, Yu S, Kim DH, Park NJ, Liu JJ, Jin YS, Kim KH (2023) Identification of the enantiomeric nature of 2-keto-3-deoxy-galactonate in the catabolic pathway of 3,6-anhydro-L-galactose. *Appl Microbiol Biotechnol* 107:7427–7438. <https://doi.org/10.1007/s00253-023-12807-7>
 33. Wang Y, Li PY, Zhang Y, Cao HY, Wang Y-J, Li CY, Wang P, Su HN, Chen Y, Chen XL, Zhang YZ (2020) 3,6-Anhydro-L-Galactose dehydrogenase VvAHGD is a member of a new aldehyde dehydrogenase family and catalyzes by a novel mechanism with conformational switch of two catalytic residues cysteine 282 and glutamate 248. *J Mol Biol* 432:2186–2203. <https://doi.org/10.1016/j.jmb.2020.02.008>
 34. Lee S, Kim KH, Kim HY, Choi IG (2017) Crystal structure analysis of 3,6-anhydro-L-galactonate cycloisomerase suggests emergence of novel substrate specificity in the enolase superfamily. *Biochem Biophys Res Commun* 491:217–222. <https://doi.org/10.1016/j.bbrc.2017.07.080>
 35. Hobbs ME, Williams HJ, Hillerich B, Almo SC, Raushel FM (2014) L-galactose metabolism in *Bacteroides vulgatus* from the human gut microbiota. *Biochemistry* 53:4661–4670. <https://doi.org/10.1021/bi500656m>
 36. Wang Y, Liu T, Guo S, Zhang P, Sun P, Chen M, Ming H (2018) Characterization and overexpression of a glycosyl hydrolase family 16 β -agarase Aga0917 from *Pseudoalteromonas fuliginea* YTW1-15-1. *J Gen Appl Microbiol* 64:276–283. <https://doi.org/10.2323/jgam.2018.02.002>
 37. Li T, Cui N, Fan J, Liu X, Guan J, Zhang W, Li Y, Gao Q, Liu Y, Xu Y, Wang L, Guo C, Wang Y (2022) Engineering β -agarase Aga0917 from *Pseudoalteromonas fuliginea* YTW1-15-1 to improve the thermostability and enzyme activity. *ACS Sustainable Chem Eng* 10:15437–15449. <https://doi.org/10.1021/acssuschemeng.2c04531>
 38. Chun J, Oren A, Ventosa A, Christensen H, Arahal DR, Da Costa MS, Rooney AP, Yi H, Xu XW, De Meyer S, Trujillo ME (2018) Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes. *Int J Syst Evol Microbiol* 68:461–466. <https://doi.org/10.1099/ijsem.0.002516>
 39. Schultz-Johansen M, Bech PK, Hennessy RC, Glaring MA, Barbeyron T, Czjzek M, Stougaard P (2018) A novel enzyme portfolio for red algal polysaccharide degradation in the marine bacterium *Paraglaciicola hydrolytica* S66^T encoded in a sizeable polysaccharide utilization locus. *Front Microbiol* 9:839. <https://doi.org/10.3389/fmicb.2018.00839>

40. Kang JY, Song HY, Kim JM (2023) Agarolytic pathway in the newly isolated *Aquimarina* sp. bacterial strain ERC-38 and characterization of a putative β -agarase. *Mar Biotechnol* 25:314–327. <https://doi.org/10.1007/s10126-023-10206-7>
41. Li C, Li C, Li L, Yang X, Chen S, Qi B, Zhao Y (2020) Comparative genomic and secretomic analysis provide insights into unique agar degradation function of marine bacterium *Vibrio fluvialis* A8 through horizontal gene transfer. *Front Microbiol* 11:1934. <https://doi.org/10.3389/fmicb.2020.01934>
42. Jamali H, Akrami F, Layeghkhavidaki H, Bouakkaz S (2025) Bacterial protein secretion systems: Mechanisms, functions, and roles in virulence. *Microb Pathog* 206:107790. <https://doi.org/10.1016/j.micpath.2025.107790>
43. Jeon EJ, Choi JW, Cho MS, Jeong KJ (2021) Enhanced production of neoagarobiose from agar with *Corynebacterium glutamicum* producing *exo*-type and *endo*-type β -agarases. *Microb Biotechnol* 14:2164–2175. <https://doi.org/10.1111/1751-7915.13899>
44. Akhtar AA, Turner DP (2022) The role of bacterial ATP-binding cassette (ABC) transporters in pathogenesis and virulence: Therapeutic and vaccine potential. *Microb Pathog* 171:105734. <https://doi.org/10.1016/j.micpath.2022.105734>
45. Drew D, North RA, Nagarathinam K, Tanabe M (2021) Structures and general transport mechanisms by the major facilitator superfamily (MFS). *Chem Rev* 121:5289–5335. <https://doi.org/10.1021/acs.chemrev.0c00983>
46. Roth P, Jeckelmann J-M, Fender I, Ucurum Z, Lemmin T, Fotiadis D (2024) Structure and mechanism of a phosphotransferase system glucose transporter. *Nat Commun* 15:7992. <https://doi.org/10.1038/s41467-024-52100-3>
47. Zhang Y, Dong S, Chen G, Cao S, Shen J, Mei X, Cui Q, Feng Y, Chang Y, Wang Y, Xue C (2023) Structural characterization on a β -agarase Aga86A_Wa from *Wenyngzhuangia aestuarii* reveals the prevalent methyl-galactose accommodation capacity of GH86 enzymes at subsite – 1. *Carbohydr Polym* 306:120594. <https://doi.org/10.1016/j.carbpol.2023.120594>
48. Anggraeni SR, Ansorge-Schumacher MB (2021) Characterization and modeling of thermostable GH50 agarases from *Microbulbifer elongatus* PORT2. *Mar Biotechnol* 23:809–820. <https://doi.org/10.1007/s10126-021-10065-0>
49. Zhang P, Zhang J, Zhang L, Sun J, Li Y, Wu L, Zhou J, Xue C, Mao X (2019) Structure-based design of agarase AgWH50C from *Agarivorans gilvus* WH0801 to enhance thermostability. *Appl Microbiol Biotechnol* 103:1289–1298. <https://doi.org/10.1007/s00253-018-9540-1>
50. Zhao H, Lee J, Chen J (2022) The hemolysin a secretion system is a multi-engine pump containing three ABC transporters. *Cell* 185:3329–3340e13. <https://doi.org/10.1016/j.cell.2022.07.017>

Figures

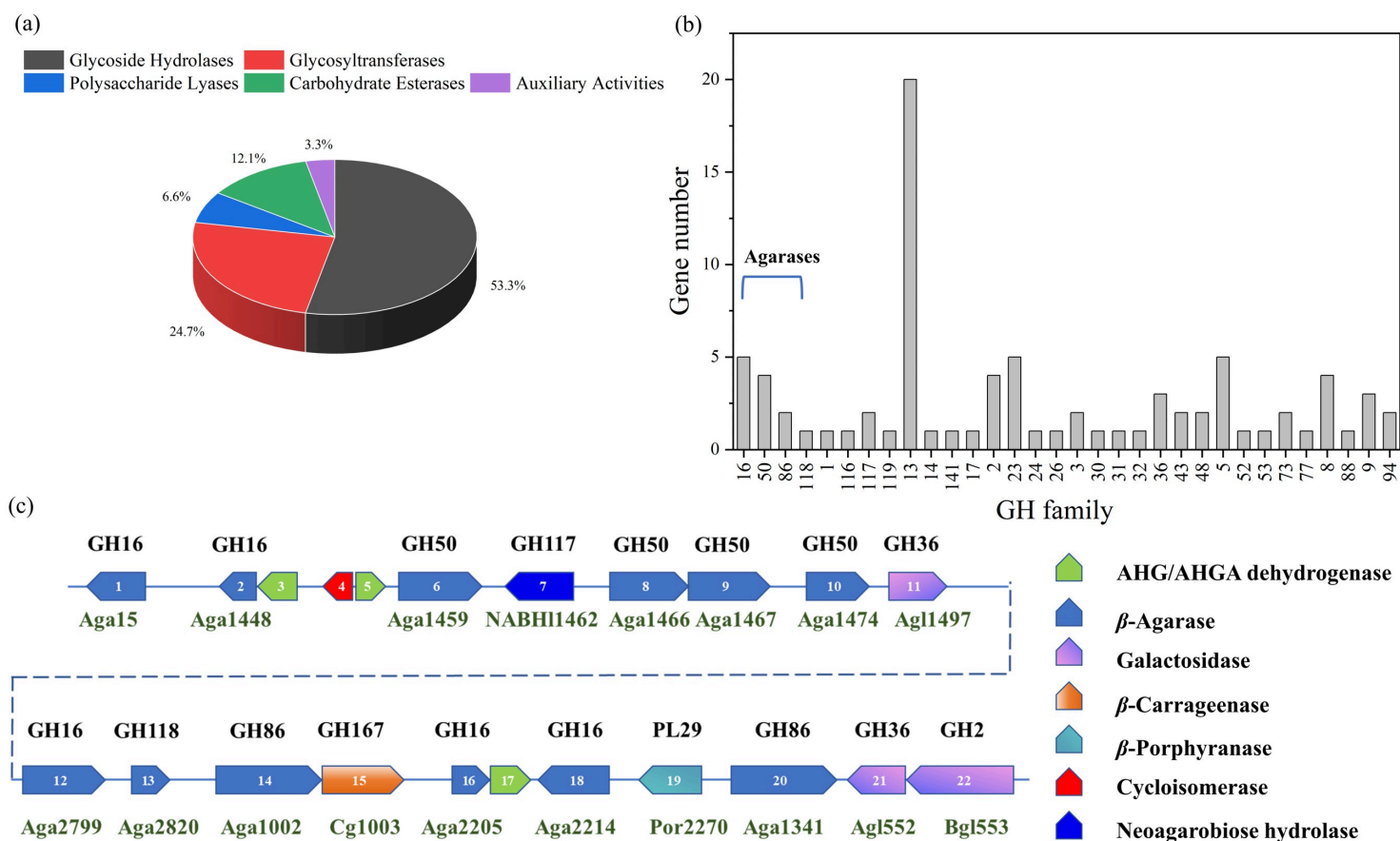


Figure 1

Genes related to the carbohydrate metabolism in the genome of *Agarivorans* sp. D1326. (a) Proportion of genes in each CAZy class; (b) The number glycoside hydrolases, specifically in families GH16, GH50, GH86, and GH118 agarases; (c) The genomic organization of a region encodes enzymes for agar degradation. Genes are numbered 1 to 22, with arrow orientations indicating direction and lengths representing the relative number of bases. Functions and family classifications were predicted using the dbCAN3 server, with colors distinguishing categories. Protein names are below the arrows, and enzyme family classifications are above

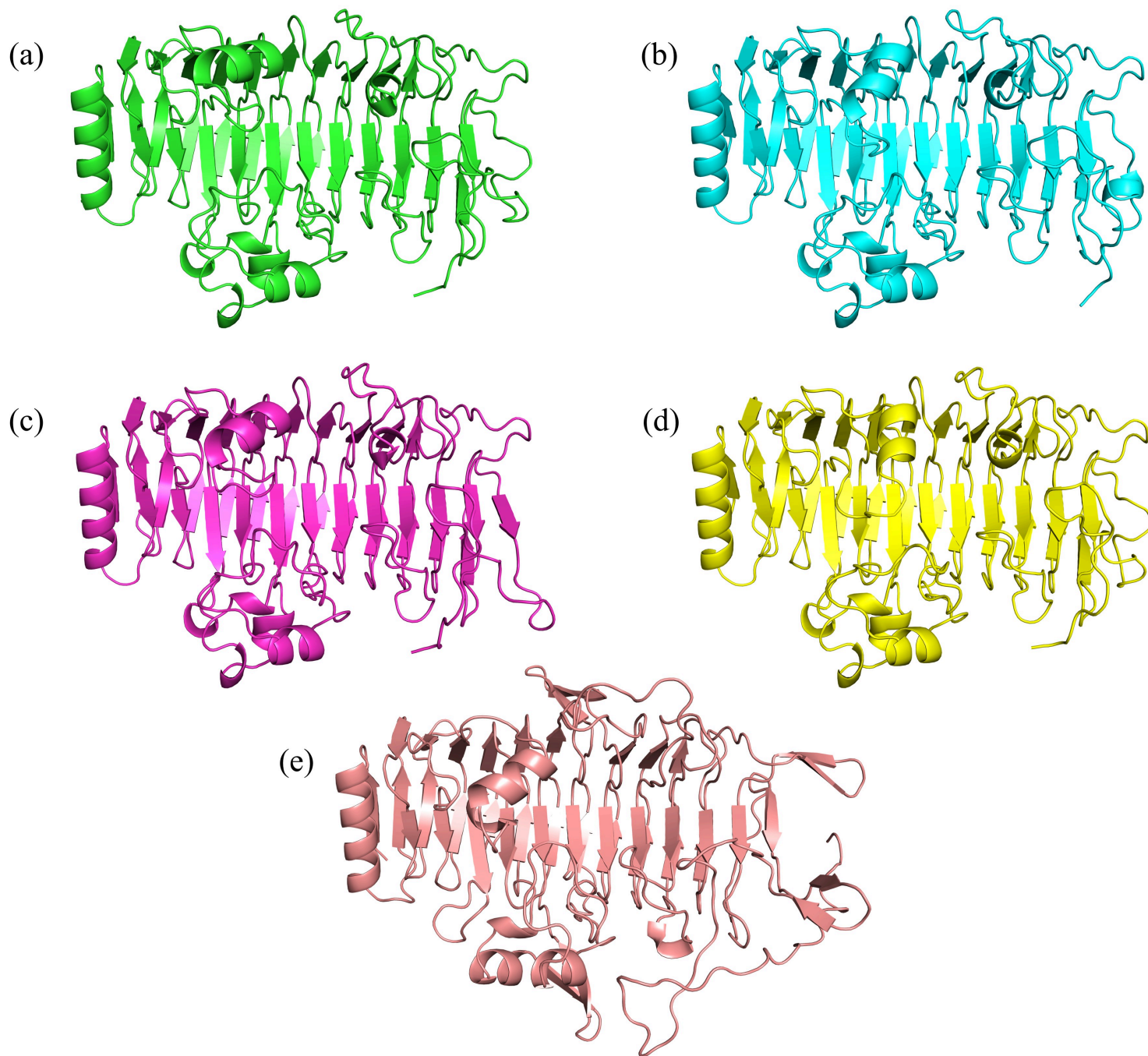


Figure 2

Structural modeling of GH118 β -agarases using AlphaFold3. (a) Aga2820 in this study; (b) AgaB (UniProt ID: AAQ56237) from *Pseudoalteromonas* sp. CY24 [11]; (c) AgaC (Q0KKW9) from *Vibrio* sp. PO-303 [12]; (d) AgaJA2 (K7WTF8) from *Agarivorans* sp. JA-1 [13]; (e) AgaXa (ADF46235) from *Catenovulum* sp. X3 [10].

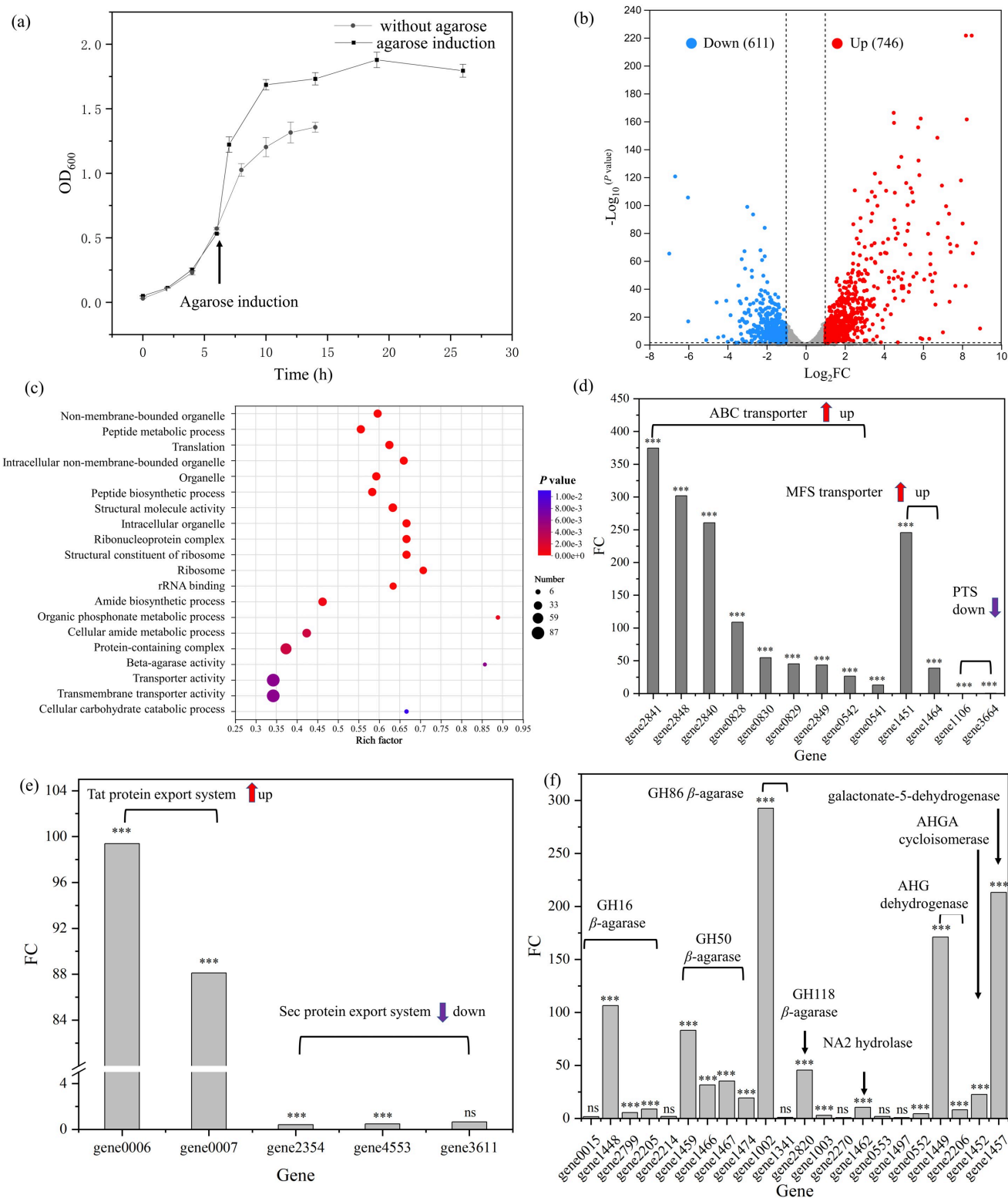


Figure 3

Analysis of gene expression level changes in *Agarivorans* sp. D1326 after induction with agarose added to the culture medium. (a) *Agarivorans* sp. D1326 was cultured in LB medium for 6 h, transferred to medium containing 0.1% agarose, cultivated for an additional 4 h, and cells were harvested. Comparative transcriptomic and proteomic analyses were performed between these cells and cells collected at the onset of induction (0 h); (b) Volcano plot analysis revealed differentially expressed genes (DEGs) before

and after agarose induction. Red dots denote upregulated genes; blue dots represent downregulated genes. FC stands for the fold change in gene expression level; (c) KEGG enrichment of top 20 relevant pathways in response to agarose induction; (d) Changes in the expression of transporter activity related genes were observed. *** indicates significant differential expression; red arrows indicate upregulation, and blue arrows indicate downregulation; (e) Expression in protein transport pathway genes; "ns" denotes non-significant differences; (f) Expression alterations in agarose metabolic genes.

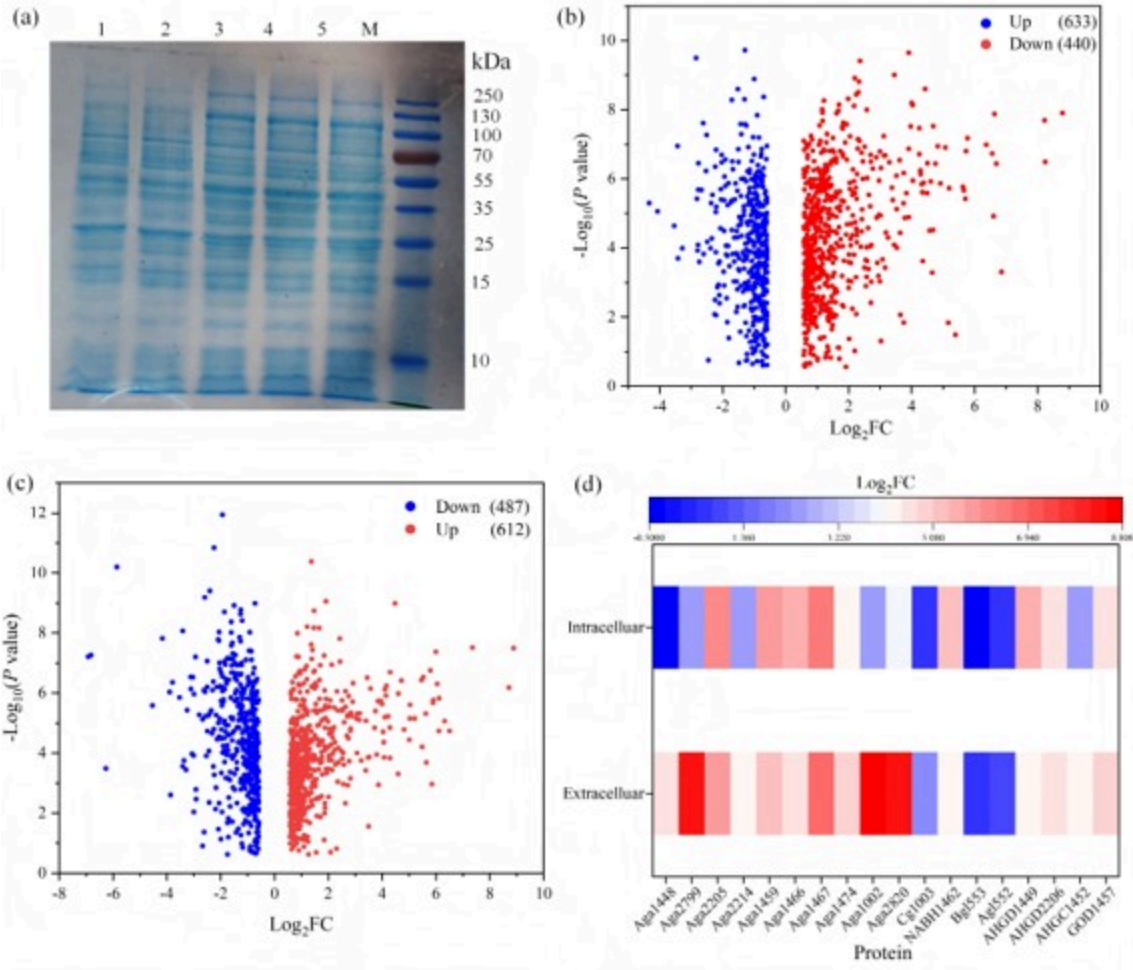


Figure 4

Proteomic profiling reveals agarose-induced expression of agarolytic enzymes in *Agarivorans* sp. D1326. (a) SDS-PAGE analysis of intracellular protein profiles pre- and post-agarose induction. Lane M: Protein marker; Lanes 1-2: Uninduced control; Lanes 3-5: Agarose-induced samples; (b) Comparative analysis of extracellular proteome profiles prior to and following agarose induction; (c) Analysis of differential protein expression in the intracellular proteome induced by agarose; (d) Heatmap visualization of agarose-induced expression changes in agarolytic enzymes across intracellular and extracellular proteomes.

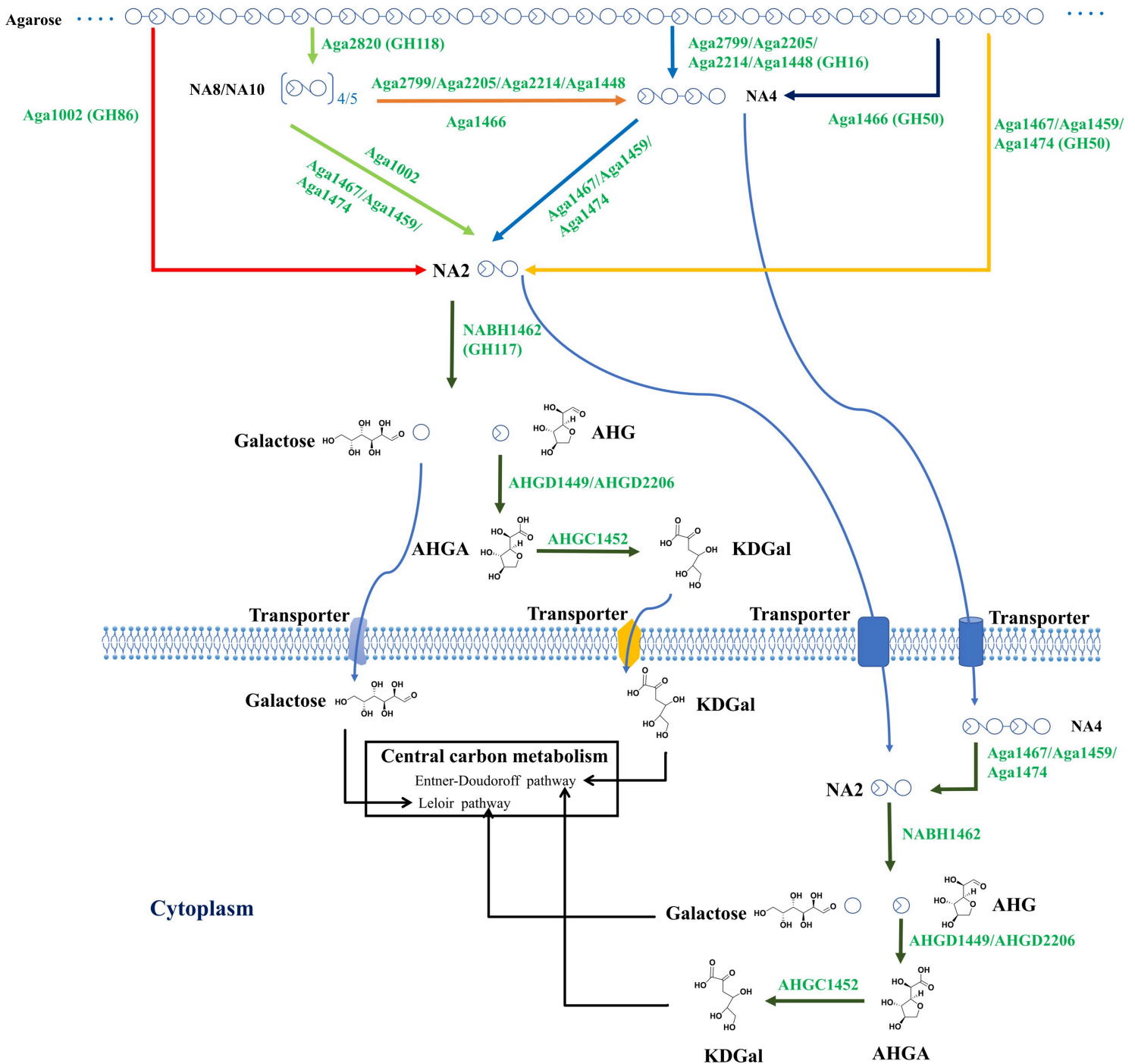


Figure 5

Proposed agarose utilization pathway in *Agarivorans* sp. D1326. Extracellular agarose undergoes three degradation routes: (1) direct conversion to NA2 by GH86 Aga1002 or GH50 Aga1467/Aga1459/Aga1474; (2) cleavage to NA8/NA10 by GH118 Aga2820 followed by degradation to NA2 via Aga1002 or GH50 enzymes; (3) hydrolysis to NA4 by GH16 Aga2799/Aga2205/Aga2214/Aga1448 or GH50 Aga1466. NA4 is further degraded to NA2 or co-transported with NA2 into cells. NA2 is hydrolyzed by NABH1462 into galactose and AHG, with AHG

metabolized both intra- and extracellularly via AHGD1449/AHGD2206 and AHGC1452 to KDGal, which enters central metabolism.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SI20260318omicscurrentmicrobiology0408.docx](#)