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Research Article

MICROBIAL MUTUALISM IN BORING CLAMS (*TRIDACNA CROCEA)* :ALLY SHORING OF OCEANS

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ABSTRACT

Ocean ecosystems are highly effective in the recycling of energy and matter. Carbon fixation is almost recycled because net carbon burial in terrestrial systems and export to the ocean via rivers. Heterotrophs efficiently reprocess organic matter because they depend on the energy in organic matter. Withal, heterotrophs cannot use total organic energy because some is shunted into metabolites like ammonium, and under anoxic conditions into reduced substances such as sulphide. These reduced inorganic compounds are used by chemo (litho) autotrophs to obtain energy for inorganic carbon fixation. Host - associated microbial symbionts are critical to the conversion of inorganic carbon into organic biomass (Beinart, R.A., 2019). In the world's oceans, Boring clams belongs to family Teredinidae, (Shipworms) with habitat of eating wood, assisted by cellulases from the intracellular symbiotic gammaproteobacteria that inhabit their gills. Other shipworms (*Kuphus polythalamius*) also relying on gill-dwelling gammaproteobacteria for sulphur oxidation (Altamia *et al*., 2020) and Methane Oxidation. The Symbionts of the gills Teredinibacter turnerae T7901 and similar strains are among the greatest sources of Biosynthetic Gene Clusters (BGCs), with content equivalent to well-known commercial manufacturers such as Streptomyces spp. This implies that shipworms might be a good source of new compounds for drug discovery (Altamia *et al*., 2020).

Keywords: Boring clams, Microbial symbionts, Sulphur oxidation, Xylotrepetic, Xylotrophic.

INTRODUCTION

Ocean ecosystems are highly effective in the recycling of energy and matter. Most of the carbon fixed is recycled because net carbon burial in terrestrial systems and export to the ocean via rivers (Cole *et al.,* 2007).Heterotrophs efficiently reprocess organic matter because they depend on the energy in organic matter. Withal, heterotrophs cannot use total organic energy because some is shunted into metabolites like ammonium, and under anoxic conditions into reduced substances such as sulphide. These reduced inorganic compounds are used by chemo (litho) autotrophs to obtain energy for inorganic carbon fixation (Raven, 2009). In the world's oceans, Boring clams belongs to family Teredinidae, (Shipworms) with habitat of eating wood, assisted by cellulases from the intracellular symbiotic gammaproteobacteria that inhabit their gills. Others which utilized sulphide metabolism relying on gill-dwelling γ-proteobacteria for sulphur oxidation. Shipworm gill symbionts of various species are thus critical for shipworm feeding and survival. One of the most interesting aspects of the shipworm system is that wood digestion does not occur in the same location as the bacteria, thus bacterial cellulose products are moved from the gill to a relatively sterile cecum, where wood digestion occurs. γ-Proteobacteria of Orders Cellvibrionales (Teredinibacter and allies) and Chromatiales (Thiosocius and allies) are described symbionts that live intracellular in the gills of shipworms.(Altamia *et al*., 2019). While many nutritional symbioses are difficult to produce, shipworm gill symbiotic proteobacteria have been successfully cultured. As a result, scientists discovered that these bacteria are excellent suppliers of secondary metabolites. Teredinibacter turnerae T7901 and similar strains are among the greatest suppliers of biosynthetic gene clusters (BGCs) among bacteria with sequenced genomes, with richness equivalent to well-known commercial manufacturers such as Streptomyces spp.

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The boring clam is the smallest of the Tridacninae subfamily, with a maximum shell size of 15 cm (6 in). It features two thick valves connected by a hinge that is normally one-third to less than half the width of the shell. Shells are often slightly to moderately elongated, and the animal is heavily inflated, particularly around the hinge.

The top valve features six to ten flattish folds that interlock at the edge with similar folds on the lower valve, allowing the shell to seal firmly. The lower valve features openings through which the byssal threads that hold the animal to the seafloor emerge.

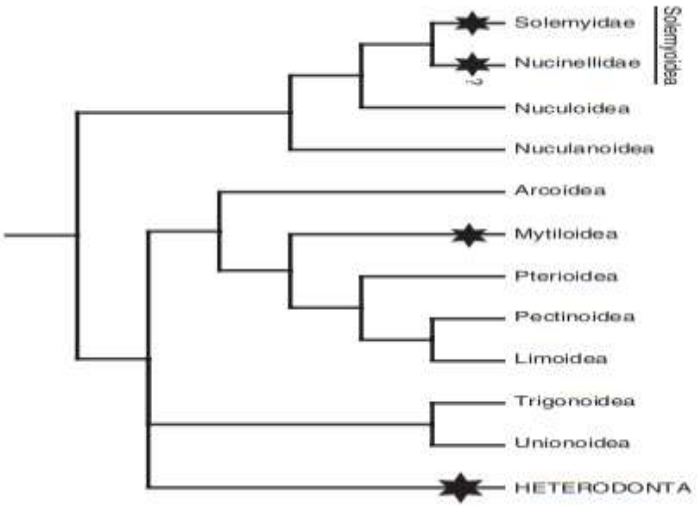


Figure 1. Phylogenetic tree of bivalve superfamilies and Heterodonta families. Withstar chemosymbionts and with triangle cellulolytic symbionts.

Microbial Rationale for the study of shipworm endosymbionts

The shipworm system is bioefficient in the breakdown of a wide range of lignocellulosic substrates and acts as an ideal target for the discovery of enzymes for the application in second-generation biofuels development. Bioactive compounds from marine organisms are currently underexploited (Piel, 2006) and *Teredinibacter turnerae* shows potential, both based on genome content and laboratory experiments, as a target for discovery of secondary metabolites showing activity against other bacteria. The shipworm endosymbiotic community display potential for discovery of novel antimicrobial compounds. The researchers describe a cellulolytic, dinitrogen-fixing bacterium isolated from the gill tissue of a wood-boring mollusc (shipworm) *Lyrodus pedicellatus* of the bivalve family Teredinidae, as well as 58 additional strains with similar properties isolated from the gills of 24 bivalve species representing 9 of 14 Teredinidae genera. The cells are Gram-negative, stiff rods with a single polar flagellum (0.4-0.6 x 3-6 microns). All isolates may grow chemoheterotrophically in a simple mineral medium supplemented with cellulose as the sole carbon and energy source. Xylan, pectin, carboxymethylcellulose (CMC), cellobiose and a variety of sugars and organic acids also support growth with addition to combined nitrogen when cultures are vigorously aerated, but all isolates fix dinitrogen under microaerobic conditions. The pH, temperature and salinity optima for growth were determined are approximately 8.5, 30-35 degrees C and 0.3 M NaCl respectively. The isolates are marine. In addition to NaCl, growth requires elevated concentrations of Ca2+ and Mg2+ that reflect the chemistry of seawater. The DNA G+C content ranged from 49 to 51-molpercentage. Based on morphological, physiological and phylogenetic characteristics and specific symbiotic association with teredinid bivalves, a new genus and species, *Teredinibacter turnerae* gen. nov., sp. nov., is proposed. The type strain is T7902 (T) (= ATCC 39867(T) = DSM 15152(T)).

Lignocellulose carbon cycling

Teredinids also called as shipworms, which are marine, obligate wood boring (xylotrepetic) and wood-feeding (xylotrophic) bivalves. As the primary consumers of woody materials across the world's oceans, shipworms play a critical role in liberating and cycling the recalcitrant carbon sequestered in lignocellulosic material, from shallow coastal driftwoods to deep-sea wood falls and mangrove stilt roots (Shipway *et al*., 2019). Shipworms use a pair of modified valves (shell-like rasps), move these with powerful adductor and foot muscles to rasp away at wood, and create a burrow (Goodell *et al.*, 2022).

Most shipworm species have a simple digestive system with a large cecum, relatively short, intestine (Figure 2). Prominent microbial communities, such as those observed in various termite hindguts, have not been observed in shipworms. In fact, the few reports available suggest that sea worms and their relatives may have few microbes in their digestive systems. In contrast, dense intracellular

communities of microbes were observed in specialized cells (bacterial cells) of the gills (Ctenidia). These endosymbionts have been shown to fix atmospheric nitrogen in a form available to the host and, when cultured in vitro, produce cellulases (enzymes that depolymerize cellulose into shorter oligocellosaccharides) and other lignocellulose-reactive enzymes. It has therefore been suggested that these symbionts are potential sources of cellulolytic enzymes that aid in the digestion of wood. Bacterial endosymbionts found in shipworms secrete CAZymes, which are found in the gill plates and ciliated epithelium along with food particles. Bacteria, prokaryotic her CAZymes, and food particles are guided into the food groove and carried towards the mouth and stomach by the movement of surface cilia. In the stomach, the crystals crush incoming food particles and bacteria with the gastric shield, effectively releasing more CAZyme and mixing them with endogenous enzymes from the animal's digestive glands. A mixture of eukaryotic and prokaryotic CAZymes reaches the cecum, where they digest wood fiber.

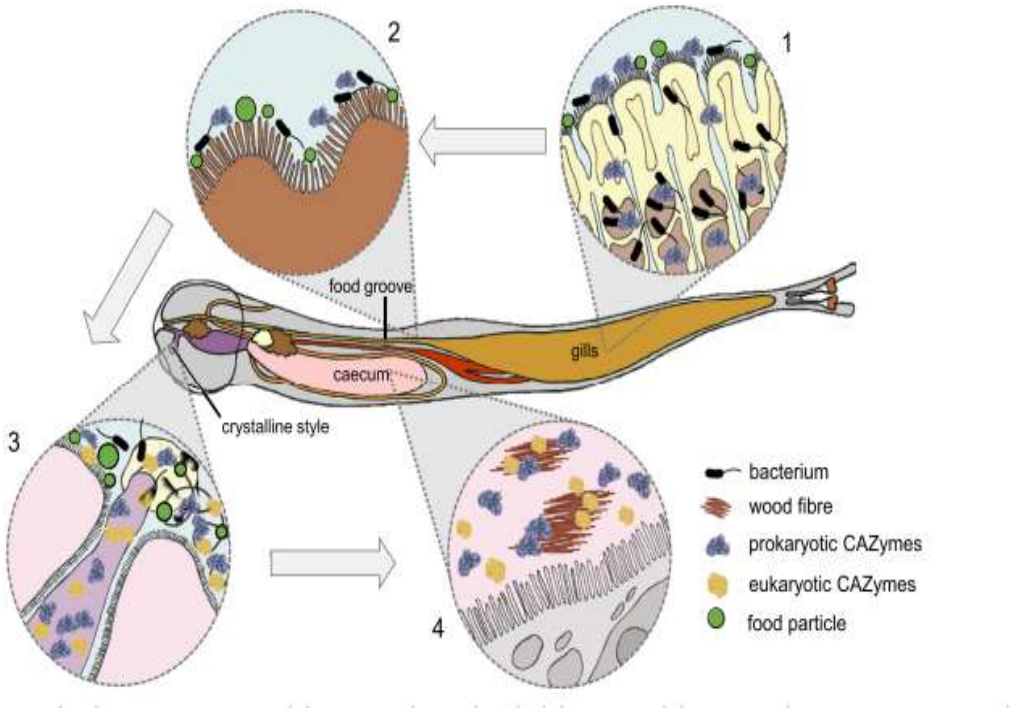


Figure 2. Digestive system of shipworms and its microbial symbiosis pathway.

Although the endosymbionts found in shipworm gills have been extensively characterized using microscopic and molecular methods, no attempt has yet been made to systematically study microbial abundance and distribution in the shipworm digestive system. Thus, the potential role of microbial fermentation in the seaworm digestive system remains largely unexplained. A Study of the digestive proteome of the shipworm *Lyrodus pedicellatus* revealed that it is dominated by a novel 300 kDa multidomain glycoside hydrolase that functions in the hydrolysis of β-1,4-glucans, and the most abundant polymers in wood. Large amounts of carbohydrate active enzymes (CAZymes) have been shown to be produced by endosymbiotic bacteria (rod-shaped bacteria)housed in specialized cells (bacteriocytes) in the animal's gills, and have been reported to play a major role in wood digestion by shipworms(Sabbadin *et al.*, 2018).Further studies revealed that there is actually not only one species of bacteria in the shipworm's gills, but that endosymbionts of many species cohabit the same host, all closely related but distinct gamma proteobacteria (Distel *et al.,* 2002).

The Wood-Boring Bivalve gills symbiosis with proteobacterial endosmbionts

Teredinibacter Turnerae, a cultured seaworm commensal species, was isolated from a variety of seaworm hosts collected from around the world (Elshahawi *et al.*,2013).This symbiotic bacterium has been shown to reside in specialized epithelial cells (bacterial cells) in the gill region of shipworms. Enzymes produced by this symbiont are proposed to facilitate the digestion of wood. Phylogenetic analysis based on 16S rRNA sequences indicated that T. turnerae is a member of the gamma subdivision of Proteobacteria. These Cells are rigid, Gram-negative rods of 0 ± 4–0 ± 6 μm inwidth and 3–6 μm in length (Distel *et al*., 2002).

Secondary metabolites in shipworms (Teredinidae)

T. turnerae T7901 genome sequence revealed nine complex polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) BGCs, and analysis is more comprehensive identifies up to 14 potential BGCs. One of

these was identified to produce a novel catecholate siderophore, turnerbactin, which is crucial in obtaining iron and to the survival of the symbiont in nature. A second BGC synthesizes the borated polyketide tartrolon, which are antibiotic and potently antiparasitic compound. Both BGCs were detected in the extracts of shipworms, implying a potential role in producing the remarkable near sterility observed in the cecum.(Elshahawi *etal*., 2013).

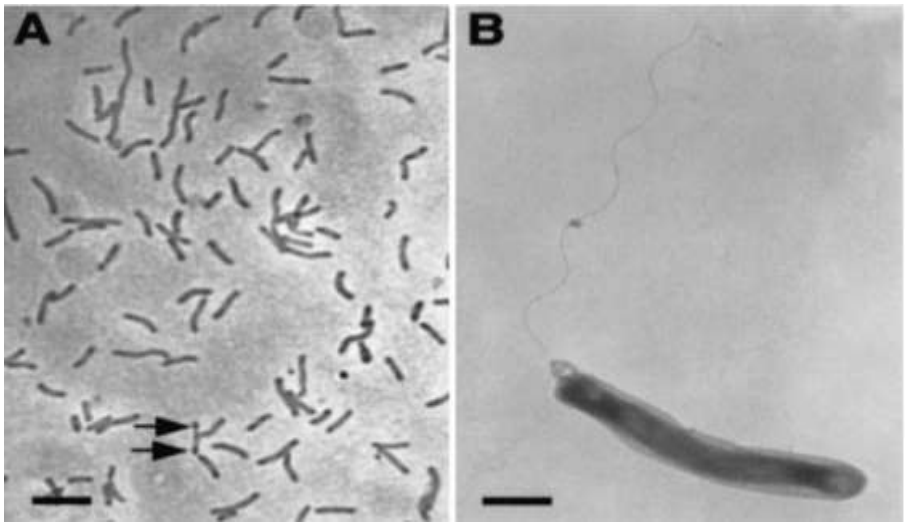


Figure 3. Proteobacterial Endosymbionts in the Gills of bivalve.

Sulphur Oxidation

Bacteria typically occur as intracellular or extracellular symbionts within specialized cells or regions of the bivalve gills. Some bacteria oxidize inorganic sulphur compounds to generate energy subsequently used to fix inorganic carbon and nitrogen into biomass that can be utilized by the host (Altamia *et al*., 2019). Proposed model of metabolism in the symbiosis between bivalve and a chemosynthetic sulfur-oxidizing bacterium (Stewart *et al*., 2005). Reduced sulphur (primarily HS^-) and NO_3^- enter the blood from the environment through unidentified transport mechanisms. CO2 and O2 enter by diffusion. In the blood, HS^- and O2 simultaneously and reversibly bind haemoglobin(Hb-O2-HS^-) for transport to the trophosome, where these substrates are used in symbiont sulfide oxidation, HS^- is oxidized first to elemental sulphur (S^0) or directly to sulphite (SO_3^{2-}). Through the APS pathway SO_3^{2-} oxidation to sulphate (SO_4^{2-}) then proceeds via the enzymes APS reductase (ii) and ATP sulfurase (iii), yielding one ATP by substrate level phosphorylation. Electrons liberated during sulphur oxidation pass through an electron transport system, driving oxygen consumption (IV) and the production of ATP and NADPH (v). Fixation of CO2 occurs primarily via ribulose 1, 5-bisphosphate carboxylase/oxygenase (Rubisco) in the Calvin Benson cycle (VI), using ATP and NADPH generated from sulphur oxidation. Transfer of organic matter from symbionts to host occurs via both translocation of simple nutritive compounds (e.g. amino acids) released by the bacteria (viii) and direct digestion of symbiont cells (ix). Several shipworm species, such as *Kuphus polythalamius*, live in other substrates. *K. Polythalamius* is common in sedimentary habitats (as well as wood), where gill symbionts are important for sulfide oxidation and carbon fixation. *K. Polythalamius* lacks significant levels of cellulolytic symbionts like *T. turnerae*, instead containing *Thiosocius teredinicola* (strain 2141T), which oxidizes sulfides to generate energy for the host. The 2141T strain is also capable of fixing atmospheric nitrogen due to a large Nif gene cluster predicted to contain molybdenum and iron acquisition genes, electron transport complex genes rnfABCDGE, core nitrogenase nifHDKT genes in a single operon and accessory genes nifQBALENXUSVPWZ. When grown autotrophically on STBA medium, strain 2141T appeared as unattached individual Gram-negative rods that are on an average, 1.7 µm long and 0.5 µm wide. Transmission electron micrographs indicate the presence of polyhedral bodies suggestive of carboxysomes and electronlucent intracellular objects similar in appearance to sulphur storage globules observed in many thioautotrophic bacteria(Altamia *et al*., 2019).

Methane oxidation

Although methane is only a minor constituent of the atmosphere, it has received considerable attention because its role as a ‘‘greenhouse gas’’. Its concentration in the atmosphere is increasing at the alarming rate of about 1% per year (Cicerone and Oremland, 1988).Microbial methane oxidation is a biogeochemical process that limits the release of methane from anaerobic environments (Hanson and Hanson, 1996). Methanotrophs are the second-most common type of chemosymbionts. These aerobic methane oxidizers use methane as an energy source as well as a carbon source (Sogin *et al*., 2021).The first

mechanism, anaerobic methane oxidation, probably involves a consortium of methanogens and sulphate-reducing bacteria, which occur universally in organic rich and anaerobic marine sediments (Kotelnikova *et al*., 2002). Duperron *et al*., (2007) detected four symbionts in the gills of Bathymodiolus heckerae including two thiotrophs, one methanotroph, and one methylotroph and found that the two sulfide-oxidizing symbionts were mutually exclusive of each other, but co-existed in bacteriocytes with the other symbiont types. Many studies have shown that anaerobic methane oxidation is important for sulfate consumption in marine sediments.

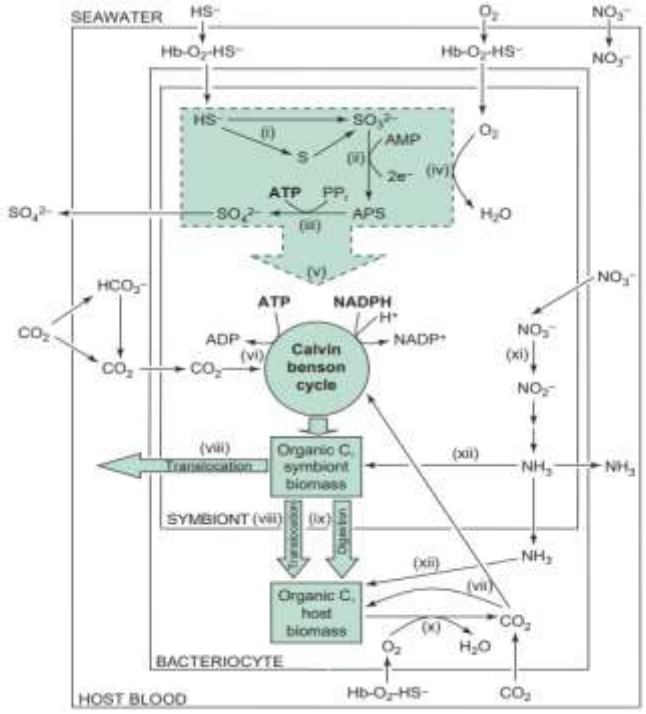


Figure 4. Sulphur oxidation in boring clams.

$$CH_4 + 2H_2O \rightarrow CO_2 + 4H_2$$

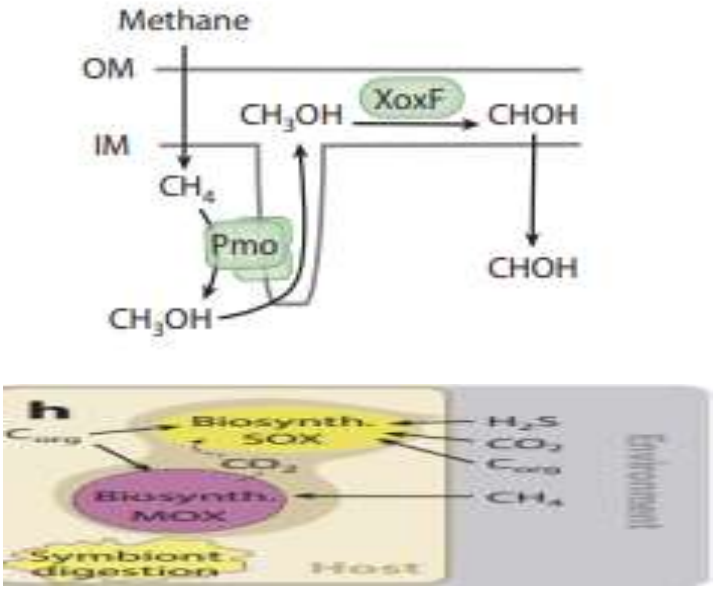


Figure 5. The metabolisms of methane oxidation (particulate methane monooxygenase a subunit).

By pumping seawater through their gills, the mussels provide the symbiont with reducing agents (reduced sulphur compounds for SOX and methane for MOX), carbon (carbon dioxide for SOX and methane for MOX), and oxygen. SOX can fix carbon dioxide, a product of methane oxidation by MOX, which also fixes carbon dioxide from seawater (Sogin et al. 2021). One to three major bacterial species predominate in seaworm gill samples, with large underlying strain variation. Metagenome sequencing was used to determine which types of bacteria inhabit gills of shipworm.

A Study on conserved biosynthetic gene cluster is regulated by quorum sensing in a shipworm symbiont (Robes Et al., 2022) which shipworm symbiont uses quorum sensing to coordinate activation of its extracellular secondary metabolism, including the transcriptional activation of a biosynthetic gene cluster that is conserved among many shipworm symbionts Bacterial symbionts often use quorum sensing (QS) to coordinate group behaviour, which is thought to help differentiate between a low-density, free-living state, and high-density, host-associated state. In many proteobacteria, Quorom S is mediated by acyl-homoserine lactone (acyl-HSL) signals produced by LuxI family synthases. In this type of QS system, genes are regulated by members of the LuxR-family of transcription factors that bind and respond to acyl-HSLs. A conserved biosynthetic gene cluster in cellulolytic shipworm symbionts is adjacent to quorum sensing genes in strain 2052S. In the genome of 2052S, the conserved BGC GCF_3 is adjacent to a luxR107 family transcription factor gene (K256DRAFT_2894, tbaR) and an acyl-HSL synthase gene (K256DRAFT_2894, tbaI). Speculated that GCF_3 may be regulated by in this strain, as is true with other QS-linked BGCs in proteobacteria.

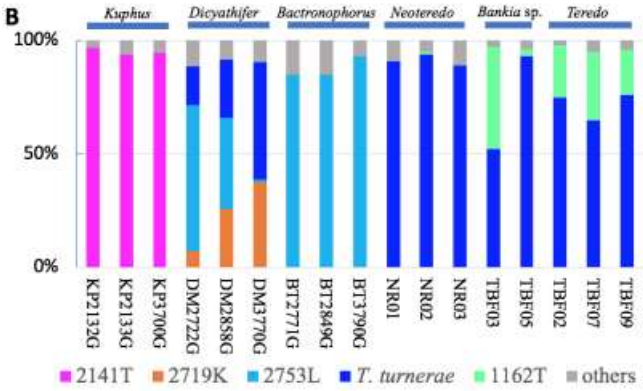


Figure 6. Metagenome analysis of shipworm gill symbionts

Phylogeny of shipworm gill symbionts and related free-living bacteria based on approximate maximum-likelihood tree of 16S rRNA sequences

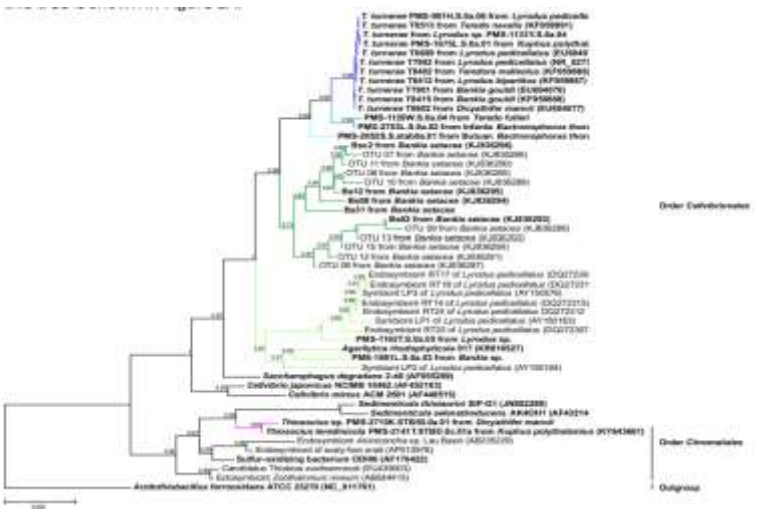
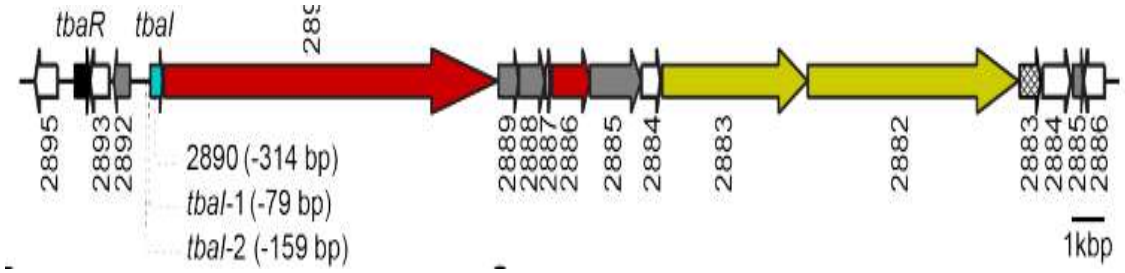
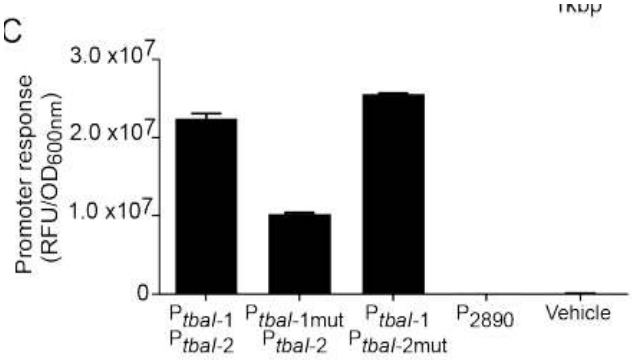


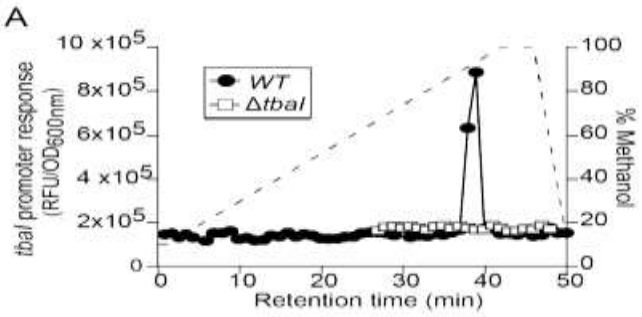
Figure 7. Phylogeny of shipworm gill symbionts.



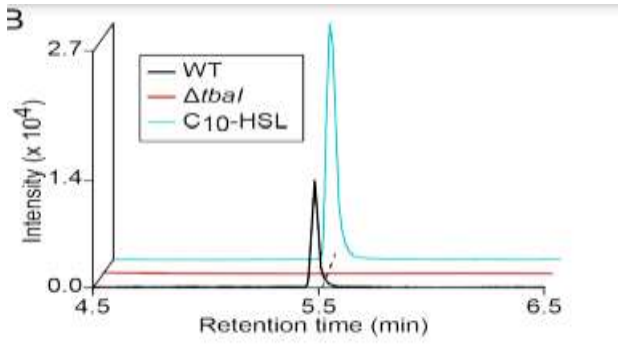
When the mutation was introduced in PtbaI-2 GFP was not affected ,suggesting that PtbaI-1 is the primary TbaR binding site. However, mutating PtbaI-1 did not completely abolish GFP activation, suggesting that TbaR can also bind PtbaI-2.



To isolate and characterize the acyl HSL signal produced by 2052S, organic supernatant extracts were separated by HPLC and each fraction was probed with the PtbaI-gfp reporter strain. This resulted in GFP fluorescence peaks in two adjacent fractions that were absent in the supernatant of in-frame unlabeled ΔtbaI mutants constructed using sucrose counterselection.

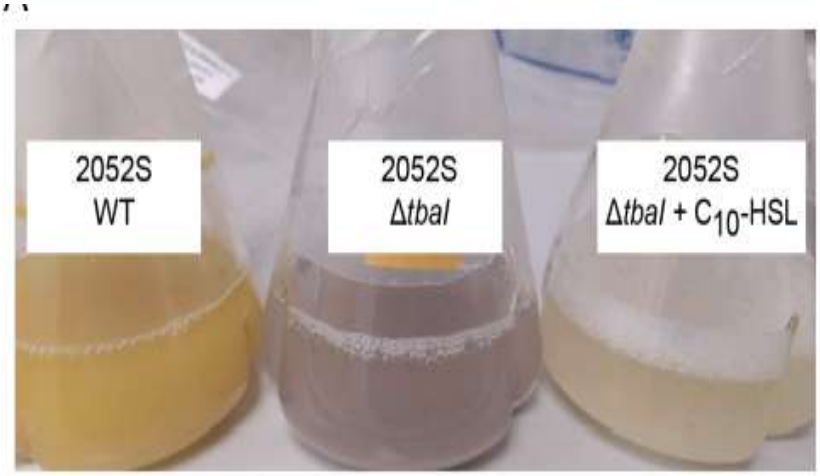


2052S produced C10-HSL by comparing WT and ΔtbaI mutant organic supernatant extracts with commercial standards of C10-HSL using high-resolution LC-MS/MS.

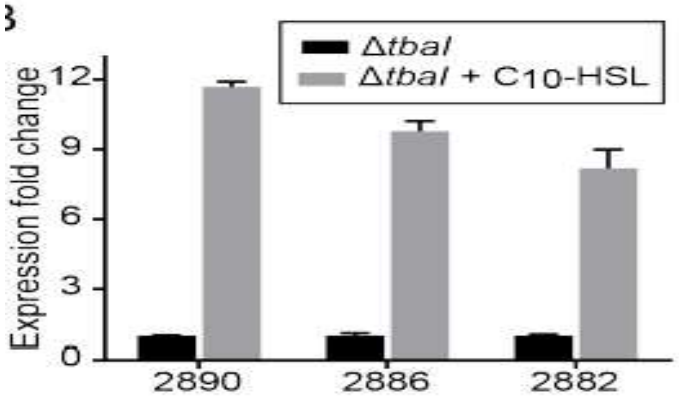


What does the 2052S regulate when using QS?

The ΔtbaI mutant was still able to utilize cellulose as a major carbon source, suggesting that cellulase production in 2052S was not regulated by QS. We found profound changes in pigmentation of ΔtbaI cultures, which could be partially compensated by adding exogenous C10-HSL. This suggests that QS may have a role in regulating the production of secondary metabolites in 2052S.



RT-PCR results show the relative expression of the GCF_3 genes K256DRAFT_2890, 2886 and 2882 after addition of C10-HSL to the ΔtbaI strain, normalized to ΔtbaI expression in the absence of signal.



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