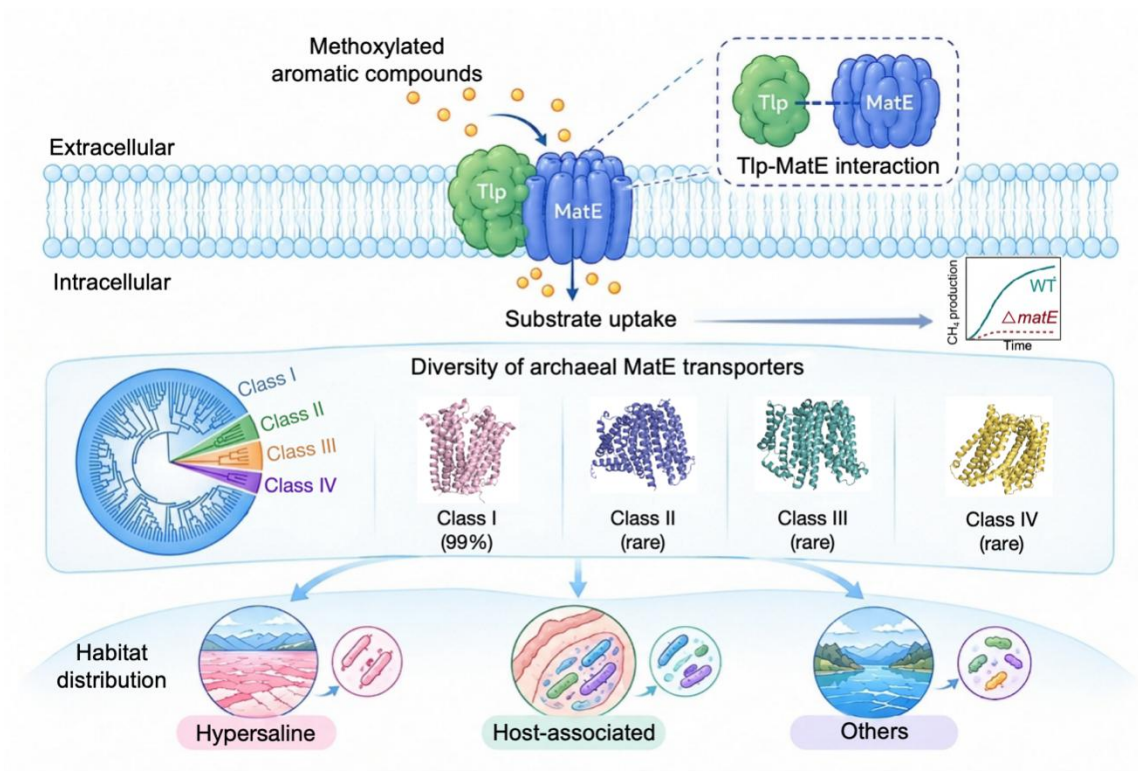


Functional characterization of a MatE transporter in *Methermicoccus shengliensis* and evolutionary diversity of archaeal MatE transporters



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**Functional characterization of a MatE transporter in
Methermicoccus shengliensis and evolutionary diversity
of archaeal MatE transporters**

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Abstract

Archaea, one of the three domains of life, exhibit remarkable physiological diversity and occupy a wide spectrum of ecological niches, ranging from extreme environments such as hypersaline lakes, hot springs, and acidic or alkaline habitats to moderate ecosystems including marine, freshwater, terrestrial, oilfield and host-associated environments. Among them, methanogenic archaea are obligate anaerobes that produce methane through distinct methanogenic pathways, traditionally utilizing simple substrates such as H_2/CO_2 , acetate, methanol, and methylated compounds. The recent discovery that *Methermicoccus shengliensis* can metabolize methoxylated aromatic compounds (MACs) has expanded the known substrate spectrum of methanogens. However, the molecular mechanisms underlying the transport of these structurally complex compounds into archaeal cells remain unknown.

In this study, we investigated the substrate transport system of *M. shengliensis* ZC-1 cultivated with methanol and 2-methoxybenzoate. Transcriptomic analysis identified two highly expressed genes, *matE* and *t1p*, potentially involved in substrate uptake. Integrating bioinformatic analyses, protein-protein interaction, and structural modeling assays, we propose a transport model comprising three sequential steps: the transduction-like protein T1p first recognizes the 2-methoxybenzoate extracellularly, then interacts with the transmembrane MATE family transporter MatE, and finally MatE mediates the translocation of the substrate into the cell. Deletion of MatE markedly impairs cell growth and methane production under methoxylated aromatic conditions, underscoring its critical role in methoxytrophic methanogenesis.

To further elucidate the diversity and habitat adaptation of archaeal MatE transporters, we performed large-scale comparative genomic phylogenetic analyses, combined with structural classification and molecular docking. Our results reveal that MatE transporters are widely distributed but unevenly represented across archaeal lineages, exhibiting structural diversity that can be classified into four major classes. Class I MatE is the most conserved and phylogenetically prevalent, whereas Classes II, III, and IV are comparatively rare. Notably, compared with MatE-negative genomes, MatE-positive archaeal genomes are significantly enriched in host-associated and hypersaline environments, as well as in heterotrophic lineages. Furthermore, specific MatE transporters exhibit strong associations with particular habitats, and their copy numbers are positively correlated with genome size. This non-random distribution indicates that the presence of MatE transporters is closely linked to specific ecological niches and metabolic strategies.

Overall, this work uncovers a previously uncharacterized transport mechanism for methoxylated aromatic compounds in methanogenic archaea and provides a

comprehensive framework for understanding the diversity and habitat adaptation of the archaeal MATE transporter family. These findings advance our understanding of substrate acquisition strategies and methane metabolism in archaea, provide new insights into their ecological distribution, and elucidate their adaptation to extreme environments, highlighting their biological importance and deepening our understanding of life on Earth.

Keywords: Archaea, Methanogens, *Methermicoccus shengliensis*, MATE family transporter, transduction-like protein, Methoxylated aromatic compounds, Methanogenesis, Methane metabolism, Comparative genomics, Environmental adaptation

Résumé

Les archées, l'un des trois domaines du vivant, présentent une remarquable diversité physiologique et occupent un large éventail de niches écologiques, allant d'environnements extrêmes tels que les lacs hypersalins, les sources chaudes et les milieux acides ou alcalins, jusqu'à des écosystèmes plus modérés incluant les milieux marins, dulcicoles, terrestres, les gisements pétroliers ainsi que les environnements associés aux hôtes. Parmi elles, les archées méthanogènes sont des anaérobies stricts qui produisent du méthane via différentes voies de méthanogenèse, utilisant traditionnellement des substrats simples tels que H_2/CO_2 , l'acétate, le méthanol et divers composés méthylés. La découverte récente selon laquelle *Methermicoccus shengliensis* est capable de métaboliser des composés aromatiques méthoxylés (MACs) a élargi le spectre de substrats connus chez les méthanogènes. Cependant, les mécanismes moléculaires permettant le transport de ces composés structurellement complexes à travers la membrane cellulaire des archées restent inconnus.

Dans cette étude, nous avons investigué le système de transport des substrats chez *M. shengliensis* ZC-1 cultivé sur méthanol et 2-méthoxybenzoate. L'analyse transcriptomique a permis d'identifier deux gènes fortement exprimés, *matE* et *tlp*, potentiellement impliqués dans l'absorption des substrats. En combinant des analyses bioinformatiques, des études d'interactions protéine-protéine et des approches de modélisation structurale, nous proposons un modèle de transport en trois étapes: la protéine de type transduction Tlp reconnaît d'abord le 2-méthoxybenzoate à l'extérieur de la cellule, puis interagit avec le transporteur transmembranaire de la famille MATE, MatE, lequel assure finalement la translocation du substrat à travers la membrane cellulaire. La délétion de MatE entraîne une diminution significative de la croissance cellulaire et de la production de méthane en présence de composés aromatiques méthoxylés, soulignant son rôle essentiel dans la méthanogenèse méthoxydotrophe.

Afin d'élucider davantage la diversité et l'adaptation aux habitats des transporteurs MatE chez les archées, nous avons réalisé des analyses phylogénétiques comparatives à grande échelle basées sur la génomique, combinées à une classification structurale et à des études de docking moléculaire. Nos résultats montrent que les transporteurs MatE sont largement distribués mais de manière inégale au sein des lignées archéennes, présentant une diversité structurale pouvant être classée en quatre grandes classes. La classe I est la plus conservée et la plus répandue sur le plan phylogénétique, tandis que les classes II, III et IV sont relativement rares. Notamment, comparés aux génomes dépourvus de MatE, les génomes archéens contenant MatE sont significativement enrichis dans les environnements associés aux hôtes et hypersalins, ainsi que dans les lignées hétérotrophes. De plus, certains transporteurs MatE spécifiques présentent de

fortes associations avec des habitats particuliers, et leur nombre de copies est positivement corrélé à la taille du génome. Cette distribution non aléatoire indique que la présence des transporteurs MatE est étroitement liée à des niches écologiques spécifiques et à des stratégies métaboliques particulières.

Dans l'ensemble, ce travail met en évidence un mécanisme de transport jusqu'alors non caractérisé pour les composés aromatiques méthoxylés chez les archées méthanogènes et propose un cadre global pour comprendre la diversité et l'adaptation écologique de la famille de transporteurs MATE chez les archées. Ces résultats améliorent notre compréhension des stratégies d'acquisition des substrats et du métabolisme du méthane chez les archées, apportent de nouveaux éclairages sur leur distribution écologique et leur adaptation aux environnements extrêmes, et soulignent leur importance biologique tout en contribuant à une meilleure compréhension de la vie sur Terre.

Mots-clés: Archées, méthanogènes, *Methermicoccus shengliensis*, transporteur de la famille MATE, protéine de type of transduction, composés aromatiques méthoxylés, méthanogenèse, métabolisme du méthane, génomique comparative, adaptation environnementale

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Chapter 4 Comparative genomic insights into MatE transporters diversity and habitat adaptation of archaea

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Chapter 4 Comparative genomic insights into MatE transporters diversity and habitat adaptation of archaea

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List of abbreviations

CH₄: Methane

CO₂: Carbon dioxide

MFR: Methanofuran

HS-CoB: Coenzyme B

HS-CoM: Coenzyme M

CoM-S-S-CoB: Heterodisulfide

F₄₂₀H₂: Reduced F₄₂₀ cofactor

MatE: MATE family transporters

Tlp: Transduction-like protein

MACs: Methoxylated aromatic compounds

PAS: Per-ARNT-Sim

PAC: PAS-associated C-terminal

MCPs: Methyl accepting chemotaxis proteins

HAMP: Histidine kinases, adenyl cyclases, methyl accepting chemotaxis proteins, and phosphatases

H₄MPT: tetrahydromethanopterin

Mtr: S-methyltransferase

Mcr: methyl-coenzyme M reductase

methyl-CoM: methyl-coenzyme M

C_{≥13}: long *n*-alkyl

C_{≥19}: *n*-alkylcyclohexanes

ABC: ATP-binding cassette

MFS: major facilitator superfamily

PACE: proteobacterial antimicrobial compound efflux

SMR: small multidrug resistance

RND: resistance nodulation cell division

AbgT: *p*-aminobenzoyl-glutamate transporter

MATE: Multidrug and Toxic Compound Extrusion

PQS: *Pseudomonas* quinolone signal

OMVs: outer membrane vesicles

T6SS: Type VI secretion system

H⁺: proton

Na⁺: sodium ion

MA: Methyl-accepting chemotaxis-like

M. shengliensis: *Methermicoccus shengliensis*

qRT-PCR: quantitative real-time PCR

LBD: Ligand-binding domain

TM: Transmembrane

TMs: transmembrane helices

SD: Signaling domain

MIC: Minimum inhibitory concentration

pac: Puromycin

ORFs: Open reading frames

RBS: Ribosome binding site

OD₆₀₀: Optical density at 600 nm

SD: Standard deviation

Al: aluminum

MSAs: Multiple-sequence alignments

MEGA11: Molecular evolutionary genetics analysis software

ML: Maximum likelihood

MOE: Molecular Operating Environment

MOP: multidrug/oligosaccharidyl-lipid/polysaccharide

PEG: polyethylene glycol

RMSD: root mean square deviation

ANOVA: One-way analysis of variance

Mb: genome size

Acr: alkyl-CoM reductases

Acs: acetyl-CoA synthase

Fd_{ox}: oxidized ferredoxin

Fd_{red}: reduced ferredoxin

Ftr: formylmethanofuran-H₄MPT formyltransferase

H₄MPT: tetrahydromethanopterin

Mch: methenyl-H₄MPT cyclohydrolase

Mer: F₄₂₀-dependent methylene-H₄MPT reductase

Mt*/Mto: methylated-specific corrinoid protein coenzyme M methyltransferase

Mtd: F₄₂₀-dependent methylene H₄MPT dehydrogenase

Pta: phosphate acetyltransferase

Chapter 1

General introduction

1.1 Context

Archaea represent the third domain of life, being more closely related to eukaryotes than to bacteria, and are characterized by unique physiological characteristics and important ecological roles (Pfeifer et al., 2021). They occupy key position in the tree of life and contribute a major fraction of microbial diversity (Adam et al., 2017). Archaea include four major superphyla: *Euryarchaeota*, TACK (an acronym for the phyla *Thaumarchaeota*, *Aigarchaeota*, *Crenarchaeota*, and *Korarchaeota*, the original phyla assigned to this group), DPANN (an acronym for the *Diapherotrites*, *Parvarchaeota*, *Aenigmarchaeota*, *Nanohaloarchaeota* and *Nanoarchaeota*, the original phyla assigned to this group), and Asgard (Baker et al., 2020; Guy et al., 2011; Seitz et al., 2019; Whitman et al., 2018). Each superphylum comprises several potentially phylum-rank clades (Evans et al., 2019; Shu et al., 2022; Spang et al., 2017), and their members are present in diverse environments, including extreme environments (high temperatures, low pH and hypersalinity) (Adam et al., 2017), oceanic, terrestrial, hot-spring, and oilfield habitats (Borrel et al., 2020; Nayfach et al., 2021). Contrary to the early assumption that archaea were mainly confined to extreme environments, they are now recognized as ubiquitous, inhabiting virtually all conceivable ecosystems on Earth and often constituting a substantial fraction of the microbial biomass (Spang et al., 2017).

Among archaea lineages, methanogenic archaea are a unique group of strictly anaerobic microorganisms with specialized metabolic capabilities and the ability to withstand extreme pressures, enabling them to thrive in extreme environments. They serve as terminal organisms in anaerobic microbial food chains and play a pivotal role in the global carbon cycle. Their distinctive metabolic process, known as methanogenesis, is believed to be an ancient and widespread pathway with both evolutionary and environmental significance. It enables the conversion of simple C1 and C2 compounds into methane to support cellular growth. Traditionally, methanogenesis has been classified into three major pathways: (1) hydrogenotrophic methanogenesis which reduce CO₂ to methane using H₂ as electron donor and is generally considered the most energy-efficient pathway, yielding approximately 0.5-1.5 mol ATP per mol of CH₄ produced, depending on the efficiency of energy conservation; (2) methylotrophic methanogenesis, which mainly use simple methyl compounds such as methanol, methylamine, and methyl sulfides as substrates for methane production, typically generating approximately 0.5-1 mol ATP per mol of CH₄; and (3) acetoclastic methanogenesis, in which the carboxyl group of acetate is oxidized to CO₂ while the methyl group is reduced to methane, yielding approximately 1 mol ATP per mol of acetate converted to methane (Buckel, 2013; Thauer et al., 2008; Welte C, 2014).

Although the biochemical pathways for methane formation from CO₂, methanol, and acetate are now well established, methanogens were long thought to rely exclusively on these simple substrates. However, recent discoveries have significantly expanded this view. In 2016, Mayumi and colleagues reported that *Methermicoccus shengliensis* ZC-1, a methanogen isolated from deep oil reservoirs, is capable of utilizing methoxylated aromatic compounds for methane production. This process has since been recognized as a fourth methanogenic pathway, termed methoxydotrophic methanogenesis (Kurth et al., 2021; Mayumi et al., 2016). Cells of *M. shengliensis* are motile cocci with diameters ranging from 0.7 to 1.0 µm (Cheng L, 2007). This metabolic capability significantly expands the known substrate spectrum of methanogenesis and challenges the long-standing assumption that methanogens are restricted to simple C1 and C2 substrates.

Despite this discovery revealed an unexpected metabolic capability in methanogens, the molecular mechanisms underlying the utilization of methoxylated aromatic compounds remain unknown. In particular, the transport processes that allow these relatively complex molecules to cross archaeal cell membranes and enter the metabolic pathways have not yet been elucidated. Understanding these transport mechanisms is therefore essential for revealing how methanogens adapt to diverse environmental substrates and ecological niches.

1.2 Objectives and research methodology

The first objective of this study was to investigate the transport of methoxylated aromatic substrates in *Methermicoccus shengliensis* and to identify potential transport components involved in this process. To address this objective, transcriptomic analysis was performed to compare the gene expression profiles of *M. shengliensis* grown with methanol and 2-methoxybenzoate as sole substrates. Differentially expressed genes potentially associated with substrate transport were subsequently identified. Quantitative real-time PCR (qRT-PCR) was used to further examine the expression patterns of selected candidate genes. Protein domain analysis was performed using SMART to characterize the predicted functions and domain organization of the candidate proteins. A bacterial two-hybrid assay was then employed to investigate potential protein-protein interactions between the identified transport-related proteins. In addition, a genetic manipulation system was established for *M. shengliensis*, and a *matE* knockout mutant was constructed to investigate the functional contribution of MatE to substrate utilization. Growth and methane production were subsequently analyzed under different substrate conditions.

The second objective was to investigate the diversity, phylogenetic distribution, and potential environmental adaptation of MatE transporters across archaeal genomes. To

address this objective, comparative genomic and phylogenetic analyses were conducted using archaeal genome datasets from diverse taxonomic groups, habitats, and metabolic types. The distribution of MatE transporters was compared with genomic characteristics, environmental habitats, and metabolic lifestyles. Structure prediction and structure-based clustering were further used to classify archaeal MatE proteins according to their predicted structural architectures. Functional annotations were compared among the different structural classes to characterize their potential functional associations. Finally, molecular docking analyses were performed to investigate the potential interactions between representative MatE structures and selected methoxylated aromatic compounds and other candidate substrates.

Overall, this study aimed to combine experimental approaches with comparative genomic, phylogenetic, and structural analyses to investigate archaeal membrane transport systems. The study specifically focused on the functional characterization of MatE in *M. shengliensis* and the evolutionary diversity of MatE transporters across archaeal genomes, providing a framework for further investigation of archaeal substrate transport and its potential ecological significance.

1.3 Chapter-by-chapter overview

Advances in methanogens

This chapter provides a systematic overview of the ecological distribution, taxonomic classification, and metabolic diversity of methanogenic archaea. It describes in detail the three classical methanogenesis pathways as well as recently discovered metabolic modes that utilize complex organic substrates, including methoxydotrophic and alkylotrophic methanogenesis. In addition, it summarizes the utilization strategies of different substrates and their ecological distribution patterns, with particular emphasis on the unresolved mechanisms of substrate uptake during the utilization of complex aromatic compounds, thereby establishing the theoretical framework and research focus of this study.

MatE transporter affects methane metabolism in *Methermicoccus shengliensis* and is modulated by methoxylated aromatic compounds

This chapter uses *Methermicoccus shengliensis* as a model organism to investigate the functional characterization and experimental validation of a MatE family transporter. Through molecular and physiological approaches, it systematically examines the role of this transporter in the uptake of methoxylated aromatic compounds and evaluates its impact on methane production efficiency. This chapter provides the first direct evidence that a MatE transporter in archaea is involved in substrate uptake and is functionally

linked to methanogenesis, thereby offering key experimental insights into the utilization of complex substrates.

Comparative genomic insights into MatE transporters diversity and habitat adaptation of archaea

This chapter systematically examines the distribution and environmental adaptability of MatE transporters in archaea from evolutionary and ecological perspectives. Based on comparative genomics and phylogenetic analyses, it characterizes the diversity of the MatE family across different archaeal lineages and explores their associations with environmental conditions and metabolic modes, thereby providing a theoretical basis for understanding the potential role of MatE transporters in archaeal ecological adaptation.

General discussion, conclusions, and perspectives

This chapter integrates and summarizes the findings of the entire study, discusses their significance for understanding methanogenic metabolic mechanisms, emphasizes the importance of membrane transport processes as a critical missing link, and outlines future research directions in transporter function, evolution, and potential biotechnological applications.

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Chapter 2

Advances in methanogens

Short overview of chapter II

Chapter II provides the scientific background for this dissertation by reviewing the diversity, metabolism, and ecological roles of methanogenic archaea. Special attention is given to the major methanogenesis pathways and the recently recognized methoxytrophic and alkylotrophic pathways that enable methanogens to utilize complex organic substrates. The chapter further highlights the poorly understood mechanisms of substrate uptake, especially for methoxylated aromatic compounds, thereby establishing the scientific rationale for investigating archaeal membrane transport systems.

2.1 The global methane cycles

Methane (CH₄) is the second most important anthropogenic greenhouse gas after CO₂, with a global warming potential approximately 25 times stronger over a 100-year time horizon and nearly 80 times greater over a 20-year period (Sobanaa et al., 2024). Although its atmospheric concentration is much lower than CO₂, CH₄ has experienced a sharp increase since the industrial revolution and continues to rise (Fowler et al., 2009; Nisbet et al., 2014; Nisbet et al., 2019). Major sources of CH₄ include both anthropogenic activities (e.g., fossil fuel extraction and use, rice cultivation, biomass burning, and wastewater treatment) and natural processes, particularly microbial production in wetlands and other anaerobic environments, which account for the majority of global emissions (Dean et al., 2018; Liu, 2008; Nazaries et al., 2013). Methane is removed mainly by oxidation with hydroxyl radicals in the atmosphere, along with minor sinks such as methanotrophy in soils and aquatic systems (Cicerone et al., 2012; Florez-Leiva et al., 2013; Li et al., 2020). Among these processes, biogenic methane production is predominantly driven by methanogenic archaea, highlighting their central role in the global methane cycle, and linking microbial metabolism with climate change.

2.2 Ecological role of biological methane production

Methane is formed by methanogenic archaea (also referred as methanogens) in the final step of organic matter fermentation in anaerobic environments. They contribute to about 1 gigaton (Gt) of the annual CH₄ production and are found in almost all types of anaerobic environments (Enzmann, 2018), ranging from hypersaline (Sorokin et al., 2017) to freshwater sediments (Borrel et al., 2012), acidic (Bräuer et al., 2006) to alkaline soils (Boone et al., 1993), invertebrate (Sprenger et al., 2000) to vertebrate gastrointestinal tracts (Dridi et al., 2012), industrial bioreactors (Ma et al., 2006), hydrocarbon-rich deposits (Mayumi et al., 2016) and rice paddies (Sakai et al., 2007) at temperatures ranging from extreme thermal to near freezing conditions (Jabłoński et al., 2015). Methanogens are a unique group of anaerobic microbes, long thought to inhabit only oxygen-free habitats, typically dying after just a few minutes of exposure to air. They are the primary contributors to global atmospheric methane emissions. Understanding methanogens is therefore crucial not only for mitigating methane emissions but also for harnessing their potential in renewable bioenergy production.

2.3 Diversity and ecological distribution of methanogenic archaea

Life on this planet would then be seen as comprising three domains, the bacteria, the archaea, and the eukaryotes, all the methanogens belong to the domain archaea (Woese et al., 1990). It is called archaea because this unique life existed on earth as early as 3.5 billion years ago (Knoll, 2015). Methanogens are capable of anaerobically fermenting inorganic or organic compounds into methane and carbon dioxide. As important environmental microorganisms, they play a vital role in the carbon cycle in nature. The currently isolated methanogens mostly belong to the *Euryarchaeota*. They can be classified into eight orders: *Methanobacteriales*, *Methanococcales*, *Methanopyrales*, *Methanomicrobiales*, *Methanocellales*, *Methanomassiliicoccales*, *Methnanonatronoarchaeales* and *Methanosarcinales* (Baptiste et al., 2005; Paul et al., 2012; Sorokin et al., 2018; Tajima et al., 2001). Furthermore, with the updates in archaeal taxonomy, especially with the development of metagenomics and high-throughput sequencing, members outside the *Euryarchaeota* are also capable of methanogenesis. And it is divided into several supergroups, including TACK (an acronym for the phyla *Thaumarchaeota*, *Aigarchaeota*, *Crenarchaeota*, and *Korarchaeota*) (Guy et al., 2011), DPANN (an acronym for the *Diapherotrites*, *Parvarchaeota*, *Aenigmarchaeota*, *Nanohaloarchaeota* and *Nanoarchaeota*) (Rinke et al., 2013) and Asgard super-phyla, all defined based on various molecular markers (Spang et al., 2017; Zaremba-Niedzwiedzka et al., 2017). Initially, *Lokiarchaeota* was the only known member of the Asgard lineage included in the TACK superphylum. However, the subsequent discovery of related lineages *Heimdallarchaeota*, *Odinarchaeota*, and *Thorarchaeota* (Williams et al., 2013; Zaremba-Niedzwiedzka et al., 2017), led to the classification of Asgard as a unique superphylum.

Specifically, 1) *Methanobacteriales* members are widely distributed across highly diverse ecological environments, such as marine and freshwater sediments, permafrost, geothermal hot springs, alkaline lakes, and the digestive tracts of various animals. The diversity of their metabolic strategies is also significant. Most members rely on CO₂ and H₂ as substrates for methane production, while a few can utilize formate, carbon monoxide, or secondary alcohols for the same purpose (Lyu, 2018). The order includes both autotrophic and heterotrophic members, with the later requiring the additional intake of acetate, amino acids, and vitamins support growth; 2) All isolated strains of the *Methanococcales* are from marine environments. They primarily use CO₂ and H₂ or formate as substrates for methane production. These archaea are known for their extremely rapid growth rate, with the representative species *Methanocaldococcus jannaschii* achieving a doubling time of 2.4 generations per hour (Jones et al., 1983); 3) *Methanopyrus kandleri*, as the only isolated culture of *Methanopyrales*, can utilize CO₂ and H₂ to produce methane. This strain has been isolated from hydrothermal vents in

multiple locations, including the Gulf of California, the Central Indian Ridge, the Mid-Atlantic Ridge, and Iceland. It is the only known archaea capable of producing methane at temperatures above 100 °C (Kurr et al., 1991; Oren, 2014); 4) Members of the order *Methanomicrobiales* are mostly mesophilic microorganisms that prefer near-neutral pH environments and are widely distributed in marine and freshwater sediments, anaerobic digesters, and rumen environments (Garcia et al., 2006). They also utilize H₂ or formate as electron donors to reduce CO₂ to CH₄, but some special members, such as *Methanofollis ethanolicus* and *Methanogenium organophilum*, are capable of methane production using primary alcohols or ethanol as substrates (Imachi et al., 2009); 5) The order *Methanocellales* mainly originates from rice soils and river sediments, and almost all members rely on the synthesis of methane from H₂ and CO₂ (Sakai et al., 2014); 6) Members of the order *Methanomassiliicoccales* are mostly chemoheterotrophic. The representative species *Methanogranum caenicola* has cocci shaped cells that occur as single cells and produces methane dependent on hydrogen and methanol (Iino et al., 2013); 7) *Methanonatronarchaeales* utilize methanol and trimethylamine as an electron acceptor, and H₂ or formate as an electron donor. They cannot grow with C1 methyl compounds alone or H₂/formate+CO₂ alone and cannot use acetate as methanogenic substrate. The representative species *Methanonatronarchaeum thermophilum* is heterotrophic and uses yeast extract as a carbon source. It is extremely halophilic, growing optimally at 4-5 M NaCl, and requires external coenzyme M as well as inoculation from anaerobic sediment from salt lakes (Sorokin et al., 2018); 8) The order *Methanosarcinales* exhibits an exceptionally broad ecological distribution, encompassing marine and freshwater sediments, animal gastrointestinal tracts, anaerobic wastewater digesters, as well as various environments such as mangroves, salt marshes, and seagrass beds, where they are consistently found in high abundance (Mand, 2019; Zhang et al., 2023). This order of archaea has the widest substrate utilization spectrum among methanogens, capable of producing methane from a variety of substrates such as CO₂/H₂, acetate, and methyl compounds (Lyu, 2018; Thauer et al., 2008). Notably, member *Methermicoccus shengliensis* of this order can use methoxylated aromatic compounds present in immature coal seams to produce methane via methoxydotrophic methanogenesis (Kurth et al., 2021).

In summary, methanogens can utilize various substrates to survive in diverse ecological environments. This diversity raises an important question: do methanogens employ the same metabolic pathways when utilizing different substrates?

2.4 Methanogenesis pathway and substrate uptake

Methanogenesis represents the central metabolic process by which methanogenic archaea produce methane as the terminal product of anaerobic organic matter degradation. Traditionally, based on substrate utilization, methanogenesis has been classified into three principal pathways: hydrogenotrophic, acetoclastic, and methylotrophic methanogenesis (Garcia et al., 2022).

Hydrogenotrophic methanogenesis, also referred to as CO₂-reducing methanogenesis, involves the reduction of CO₂ to CH₄ using H₂ or formate as electron donors. Acetoclastic methanogenesis converts acetate into CH₄ and CO₂ through a disproportionation reaction. Methylotrophic methanogenesis utilizes methylated compounds (e.g., methanol, methylamines, and methyl sulfides) and proceed via two different modes: (i) dismutation of methyl groups into CH₄ and CO₂, or (ii) reduction of methyl compounds to CH₄ using H₂ or formate as electron donors, commonly termed H₂-dependent methylotrophic methanogenesis (Garcia et al., 2022).

Recent discoveries have significantly expanded the known metabolic capabilities of methanogenic archaea. Methanogens are now recognized to utilize not only simple C1 and C2 substrates but also structurally more complex organic compounds. In 2016, Mayumi et al. reported that *Methermicoccus shengliensis* can directly convert coal-derived methoxylated aromatic compounds into methane, leading to the proposal of a fourth methanogenic pathway termed methoxydotrophic methanogenesis, in which methoxyl groups derived from complex organic matter serve as the methane precursor (Mayumi et al., 2016).

Subsequent metagenomic analyses suggested that certain archaea may also metabolize alkanes to produce methane (Borrel et al., 2019; Laso-Pérez et al., 2019). This hypothesis was experimentally confirmed by Zhou et al., who demonstrated that a single archaeon, *Candidatus Methanoliparum*, is capable of directly converting long-chain alkyl hydrocarbons (C_≥13) into methane. Using isotope labeling experiments combined with metagenomic and metatranscriptomic analyses and high-resolution mass spectrometry, the authors showed that this organism can convert hydrocarbons into methane via a previously unrecognized metabolic strategy, designated alkylotrophic methanogenesis. Together, these findings expand the classical view of methanogenic metabolism and establish five currently recognized methanogenesis pathways (Zhou et al., 2022) (**Figure 2-1**).

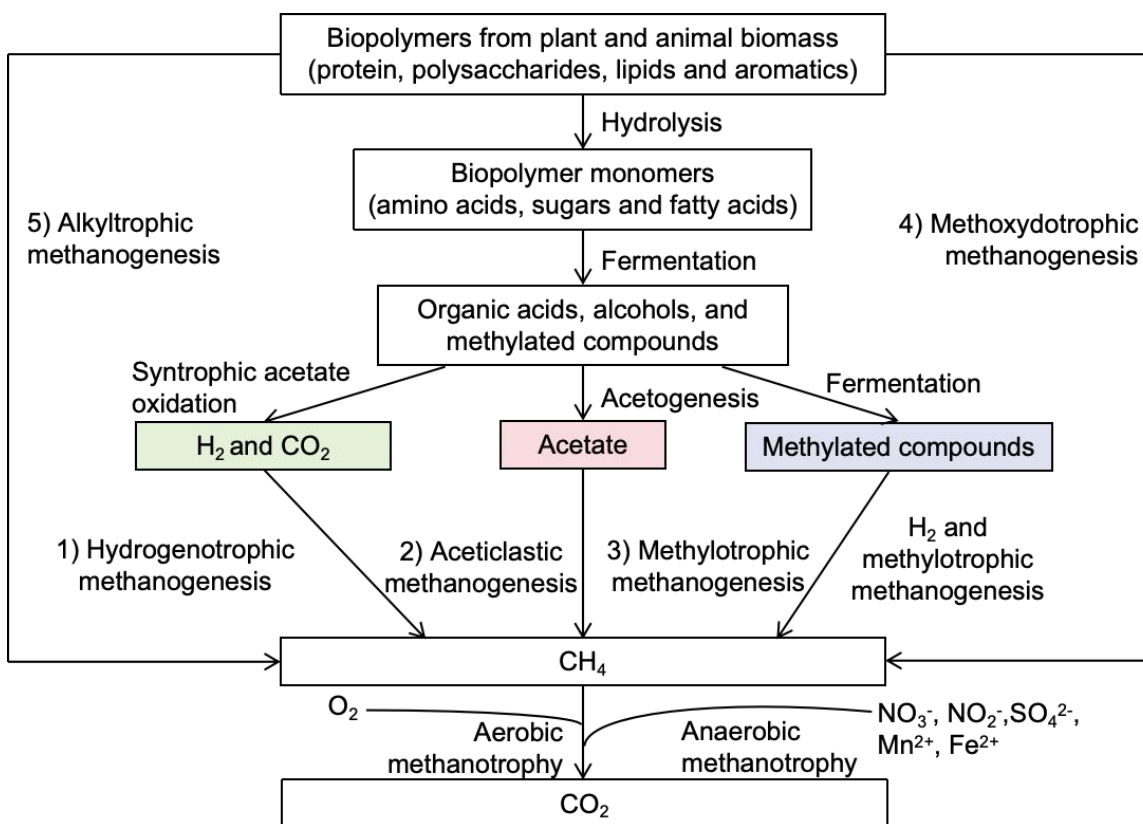


Figure 2-1. Methanogenesis in the context of the global carbon cycle. The five methanogenesis pathways are adapted from the previous papers by Evans et al., 2019, ‘An evolving view of methane metabolism in the archaea’, and Garcia et al., 2022, ‘Diversity and evolution of methane-related pathways in archaea’ (Evans et al., 2019; Garcia et al., 2022)

Despite the diversity of substrates involved in these pathways, methane production ultimately follows a highly conserved biochemical framework. Methanogens share a conserved core methanogenic pathway consisting of seven enzymatic steps (**Figure 2-2**). The first five steps overlap with the archaeal Wood-Ljungdahl (WL) pathway, during which CO_2 is sequentially reduced to methyl-tetrahydromethanopterin ($\text{CH}_3\text{-H}_4\text{MPT}$). The process begins with the reduction of CO_2 to formyl-methanofuran (CHO-MFR), catalyzed by formylmethanofuran dehydrogenase (Fwd) using reduced ferredoxin (Fd_{red}) as the electron donor. The formyl group is then transferred to tetrahydromethanopterin (H_4MPT) by formyltransferase (Ftr). The formyl bound to H_4MPT is stepwise reduced into methenyl-, methylene-, and methyl- levels by methenyl- H_4MPT cyclohydrolase (Mch), F_{420} -dependent methylene- H_4MPT dehydrogenase (Mtd), and F_{420} -dependent methylene- H_4MPT reductase (Mer), ultimately forming $\text{CH}_3\text{-H}_4\text{MPT}$ (Evans et al., 2019).

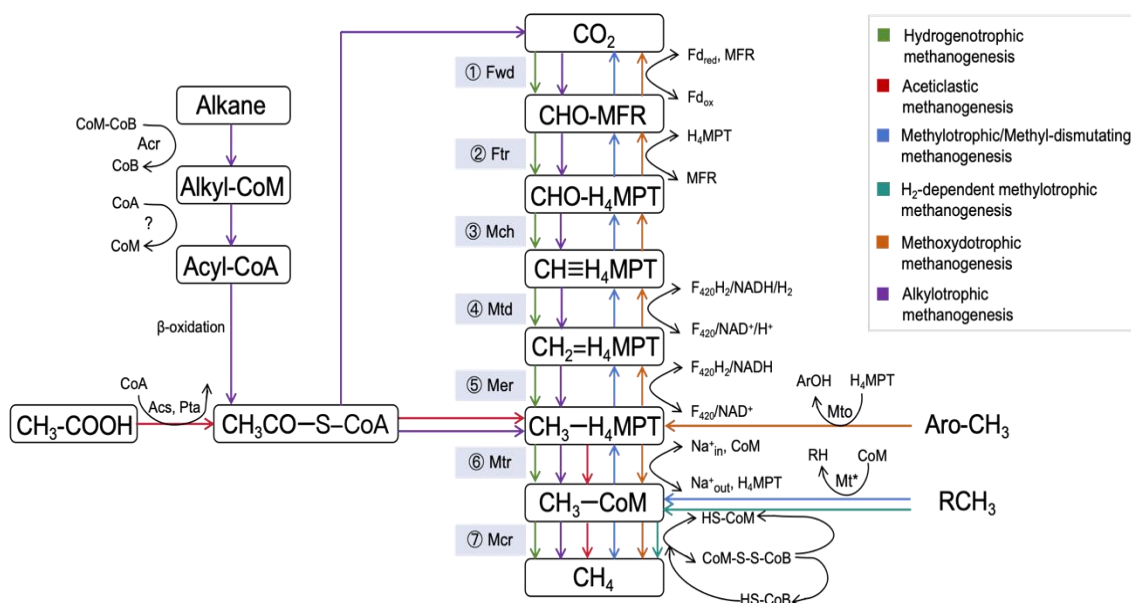


Figure 2-2. Currently known methane-related pathways are adapted from the previous papers by Evans et al., 2019, ‘An evolving view of methane metabolism in the Archaea’, and Garcia et al., 2022, ‘Diversity and evolution of methane-related pathways in archaea’ (Evans et al., 2019; Garcia et al., 2022). Arrows are colored according to the different pathways indicated in the figure. Acr, alkyl-CoM reductases; Acs, acetyl-CoA synthase; CoB-SH, coenzyme B; HS-CoM, coenzyme M; Fd_{ox}, oxidized ferredoxin; Fd_{red}, reduced ferredoxin; Ftr, formylmethanofuran-H₄MPT formyltransferase; Fwd, formylmethanofuran dehydrogenase; H₄MPT, tetrahydromethanopterin; Mch, methenyl-H₄MPT cyclohydrolase; Mcr, methyl-coenzyme M reductase; Mer, F₄₂₀-dependent methylene-H₄MPT reductase; MFR, methanofuran; Mt*/Mto, methylated-specific corrinoid protein coenzyme M methyltransferase; Mtd, F₄₂₀-dependent methylene H₄MPT dehydrogenase; Mtr, Na⁺-translocating methyl-H₄MPT-coenzyme-M methyltransferase; Pta, phosphate acetyltransferase.

In the final stages of methane formation, the methyl group is transferred from CH₃-H₄MPT to coenzyme M (HS-CoM) by the membrane-associated methyltransferase complex (Mtr). This reaction is coupled to Na⁺ translocation across the cytoplasmic membrane, generating a sodium motive force. Subsequently, methyl-coenzyme M reductase (Mcr) catalyzes the reduction of methyl-S-CoM with coenzyme B (CoB-SH), producing methane and the heterodisulfide CoM-S-S-CoB. This final step is universally conserved and represents the biochemical hallmark of methanogenesis (Borrel et al., 2016).

In hydrogenotrophic methanogenesis, CO₂ is reduced through the core methanogenic pathway described above. Energy conservation occurs primarily during the

methyl transfer step catalyzed by the membrane bound Mtr complex, which translocates sodium ions across the membrane and generates a sodium motive force that subsequently drives ATP synthesis (Kurth et al., 2020). This form of methanogenesis is commonly observed in deep-sea sediments, termite gut systems, and the gastrointestinal tracts of animals and humans (Lyu et al., 2018). Because H₂ and CO₂ are small molecules capable of diffusing across archaeal membranes (Thauer et al., 2008), no dedicated transporters for these substrates have been identified so far.

In acetoclastic methanogenesis, acetate is first activated and converted into a methyl intermediate that enters the core methanogenic pathway. The methyl group of methyl-H₄MPT is subsequently transferred to methyl-S-CoM by Mtr, leading to Na⁺ translocation across the membrane and the formation of CoM-S-S-CoB by Mcr (Welte et al., 2014a). This metabolic strategy is primarily carried out by members of the families *Methanosarcinaceae* and *Methanotrichaceae*. Acetate uptake has been shown to occur via specific acetate transport systems (Welte et al., 2014b). For example, in *Methanosarcina mazei*, acetate transport depends on an acetate permease (MM_0903), as demonstrated through gene deletion experiments (Welte et al., 2014b). Ecologically, acetoclastic methanogens play a major role in methane production in anaerobic digesters, rice paddies, and wetland ecosystems (Lyu et al., 2018).

Methylotrophic methanogenesis is primarily performed by members of the order *Methanosarcinales* and is characterized by substrate-specific methyltransferase systems that channel methyl groups into the core pathway via methyl-CoM. These systems typically consist of three components: a substrate-specific methyltransferase that transfers the methyl group to a corrinoid protein, followed by a second methyltransferase that transfers the methyl group to coenzyme M, thereby generating methyl-CoM and feeding into the central methanogenic pathway. In this metabolism, approximately three-quarters of the methyl groups are reduced to methane, while the remaining fraction is oxidized to CO₂ to generate reducing equivalents. These electrons drive a membrane-bound electron transport chain, in which heterodisulfide serves as the terminal electron acceptor, enabling energy conservation through the generation of a proton or sodium motive force (Sorokin et al., 2017). Despite the broad range of methylated substrates utilized, the mechanisms underlying their cellular uptake remain poorly understood. Methanol is generally assumed to diffuse passively across membranes, whereas methylamines are thought to be transported via yet-unidentified permeases. Ecologically, methylotrophic methanogens are widely distributed across diverse environments, including marine systems, hypersaline habitats, the bovine rumen, and sulfate-rich sediments (Kendall et al., 2006; Sorokin et al., 2017; Thauer, 1998).

Next to the three common methanogenesis, some methanogens use a mixed mode of methanogenesis by combining the hydrogenotrophic and the methylotrophic methanogenesis, which is seen as a novel mode of energy metabolism in methanogenic archaea, named H₂-dependent methylotrophic methanogenesis. In this pathway, the methyl compound only acts as an electron acceptor, receiving electrons from H₂ or formate. Subsequently, the methyl compound is directly reduced to methane, without going through the reverse H₂/CO₂ reduction pathway. This pathway was first discovered in *Methanobacteriales* (Fricke et al., 2006) and *Methanomicrobiales* (Sprenger et al., 2007), and has since been reported in *Methanomassiliicoccales* (Borrel et al., 2014), *Methanofastidiosa* (Nobu et al., 2016), *Bathyarchaeota* (Evans et al., 2015), and *Verstraetearchaeota* (Wang et al., 2018). Recent studies speculate that this pathway is the highest Gibbs free energy methanogenesis route in natural environments. It plays a dominant role in anaerobic environments with low H₂ concentrations and is also the most primitive type of methanogenesis (Lyu et al., 2018). Like the hydrogenotrophic pathway, H₂-dependent methylotrophic methanogenesis involves small and diffusible molecules (H₂ and methyl compounds), and no dedicated transporters have yet been reported.

Beyond these classical pathways, methoxydotrophic methanogenesis represents a major conceptual advance by demonstrating that methanogens can access lignin- and coal-derived substrates (Mayumi et al., 2016). In this pathway, methyl group from methoxylated compounds is dismutated into CH₄ and CO₂. This pathway is like methyl-dismutating methanogenesis, except that the methyl group is first transferred to H₄MPT instead of HS-CoM by MtoABC methyltransferases/corrinoid complex (Kurth et al., 2021). Although *M. shengliensis* is currently the only cultivated representative, this metabolism highlights the potential importance of complex organic matter in subsurface methane cycling (Kurth et al., 2021; Mayumi et al., 2016). Notably, the mechanisms by which methoxylated aromatic compounds are transported into the cell remain unexplored.

To date, the novel archaeon *Candidatus Methanoliparum*, isolated and cultivated by our research group, has been shown to directly convert alkyl hydrocarbons into methane, and named alkylotrophic methanogenesis. The long-chain alkanes are first activated by a Mcr-like enzyme (Acr) to generate alkyl-CoM. The alkyl is then oxidized into acyl and transferred to CoA by unknown proteins, forming acyl-CoA. This intermediate is further metabolized into several acetyl-CoA by the β -oxidation pathway. The acetyl-CoA is split into CO₂ and methyl group, forming methyl-H₄MPT. Genomic analysis of *Ca. Methanoliparum* reveals the presence of genes for alkyl-coenzyme M reductase, beta-oxidation pathway, the Wood-Ljungdahl pathway, and the methyl-coenzyme M reductase pathway, supporting its capacity to directly convert long *n*-alkyl (C_{≥13}), *n*-alkylcyclohexanes (C_{≥19}), and *n*-alkylbenzene into methane, thereby facilitating

methanogenesis directly from crude oil (Zhou et al., 2022). *Ca. Methanoliparum* are present in various anoxic hydrocarbon-rich environments worldwide (Borrel et al., 2019; Laso-Pérez et al., 2019; Wang et al., 2021).

Collectively, these discoveries demonstrate that methanogens are not limited to simple C1/C2 substrates such as CO₂, methanol, or acetate but can also utilize structurally complex organic compounds. The uptake of such large and often hydrophobic molecules is unlikely to rely solely on passive diffusion, suggesting the involvement of specialized membrane transport systems. However, despite extensive research on methanogenic metabolism, the mechanisms responsible for substrate transport in methanogens remain unexplored.

2.5 *Methermicoccus shengliensis*

Methermicoccus shengliensis is a thermophilic methanogenic archaeon belonging to the order *Methanosarcinales*, a lineage characterized by high metabolic versatility. The strain *M. shengliensis* ZC-1 (DSM 18856) was isolated from oil-production water of the Shengli oilfield in China and grows optimally at 65 °C and pH 6.0-6.5 (Cheng et al., 2007). Consistent with typical methylotrophic methanogens, early physiological studies demonstrated that it utilizes simple methylated substrates such as methanol, methylamine, and trimethylamine, for methane production (Cheng et al., 2007).

In 2016, Mayumi et al. unexpectedly discovered that *M. shengliensis* can produce methane from dozens of methoxylated aromatic compounds (MACs) present in various types of coal, including 2-methoxybenzoate, 3-methoxybenzoate, 3,4,5-trimethoxybenzoate, 3,4,5-trimethoxy-cinnamate, 1,2,3-trimethoxy-benzene, and 3,4,5-trimethoxybenzylalcohol. This finding significantly expanded the known substrate spectrum of methanogenesis beyond simple C1 and C2 compounds (Mayumi et al., 2016). Notably, this pathway represents an early and direct extension of methanogenic metabolism toward structurally complex organic substrates, particularly lignin-derived aromatic compounds that are abundant in coal and subsurface environments.

Although *M. shengliensis* retains the genetic capacity for classical methylotrophic methanogenesis, where methylated substrates are disproportionated into methane and CO₂, the metabolism of methoxylated aromatic compounds follows a distinct strategy. Despite the identical oxidation state of methyl and methoxy groups, isotope-tracing experiments demonstrated that methoxydotrophic methanogenesis unprecedentedly entails both methyl disproportionation and CO₂ reduction to CH₄ (Mayumi et al., 2016), indicating the involvement of a previously unrecognized methanogenic pathway.

Building on this discovery, Kurth et al. further elucidated the metabolic basis of methoxydotrophic methanogenesis in *M. shengliensis*. Multi-omics (genomics,

transcriptomics, and proteomics) reveal that this process relies on a specialized O-demethylation/methyltransferase system targeting aromatic methoxy groups (ArOCH_3) (Kurth et al., 2021).

In anaerobic microorganisms, methyl-group metabolism typically involves a two-step transfer system in which a substrate-specific methyltransferase (MT1) transfers the methyl group to a corrinoid protein (CP), followed by transfer to a physiological C1 carrier-coenzyme M (CoM) via a second methyltransferase (MT2). In methanogenic archaea, this carrier is coenzyme M (CoM), whereas in bacteria it is tetrahydrofolate (H_4F) (Ferguson et al., 1996; Kaufmann et al., 1997; Oelgeschläger et al., 2009; Sauer et al., 1997).

In contrast, genome-centered analyses in *M. shengliensis* identified a distinct gene cluster encoding a putative methyltransferase system associated with methoxylated aromatic compound metabolism. This cluster includes O-demethylases/methyltransferase (BP07_RS03250; BP07_RS03255), a corrinoid protein (BP07_RS03260), a corrinoid activation protein (BP07_RS03235), and an MtrH-like methyltransferase (BP07_RS03240), which likely function cooperatively to transfer methyl groups derived from methoxylated aromatic compounds into the methanogenic pathway (Kurth et al., 2021). During this process, the methoxyl group is converted to methane, whereas the aromatic ring is released as the corresponding hydroxylated compound, accompanied by CO_2 formation (Kurth et al., 2021).

Importantly, unlike canonical methanogenic pathways in which methyl groups are directly transferred to coenzyme M (Ferguson et al., 1996), evidence suggests that in *M. shengliensis* ArOCH_3 methyltransferase rather uses tetrahydromethanopterin (H_4MPT) as final C₁ carrier (H_4MPT) (Kurth et al., 2021). The different entry point into methanogenic pathway via $\text{CH}_3\text{-H}_4\text{MPT}$ rather than $\text{CH}_3\text{-CoM}$ putatively triggers changes in energetics, thermodynamics, and kinetics that might involve an idiosyncratic C₁ catabolism cycling between oxidation and reduction (Kurth et al., 2021).

In addition to intracellular metabolic adaptations, substrate uptake represents a critical yet unresolved step in methoxydotrophic methanogenesis. Given that methyltransferases and corrinoid proteins are localized in the cytoplasm, *M. shengliensis* needs transporters for the uptake of methoxylated aromatic compounds (Kurth et al., 2021). Although specific transporters for such substrates have not yet been found in methanogens, accumulating evidence from bacterial systems indicates that many substrates of multidrug and toxic compound extrusion (MATE) transporters share common structural features, particularly the presence of aromatic moieties (Lu et al., 2013). Indeed, well-characterized MATE transporters, such as NorM homologs from *Neisseria gonorrhoeae* (NorM-NG) (Long et al., 2008), *Vibrio parahaemolyticus* (NorM-

VP) (Morita et al., 2000), and *Vibrio cholerae* (NorM-VC) (He et al., 2010), recognize and export structurally diverse compounds that are typically polyaromatic, including ethidium, rhodamine 6G, and tetraphenylphosphonium (Lu et al., 2013). This substrate profile suggests that MATE transporters possess an intrinsic capacity to accommodate aromatic compounds.

Notably, in *M. shengliensis*, a gene encoding a MatE family transporter (BP07_RS03225) is co-localized with the demethoxylation-associated methyltransferase gene cluster (Kurth et al., 2021), suggesting a potential functional linkage between substrate transport and downstream metabolism. This genomic context raises the possibility that MatE transporters may mediate the uptake of methoxylated aromatic compounds (Figure 2-3). However, their specific role in archaeal systems remains to be experimentally validated.

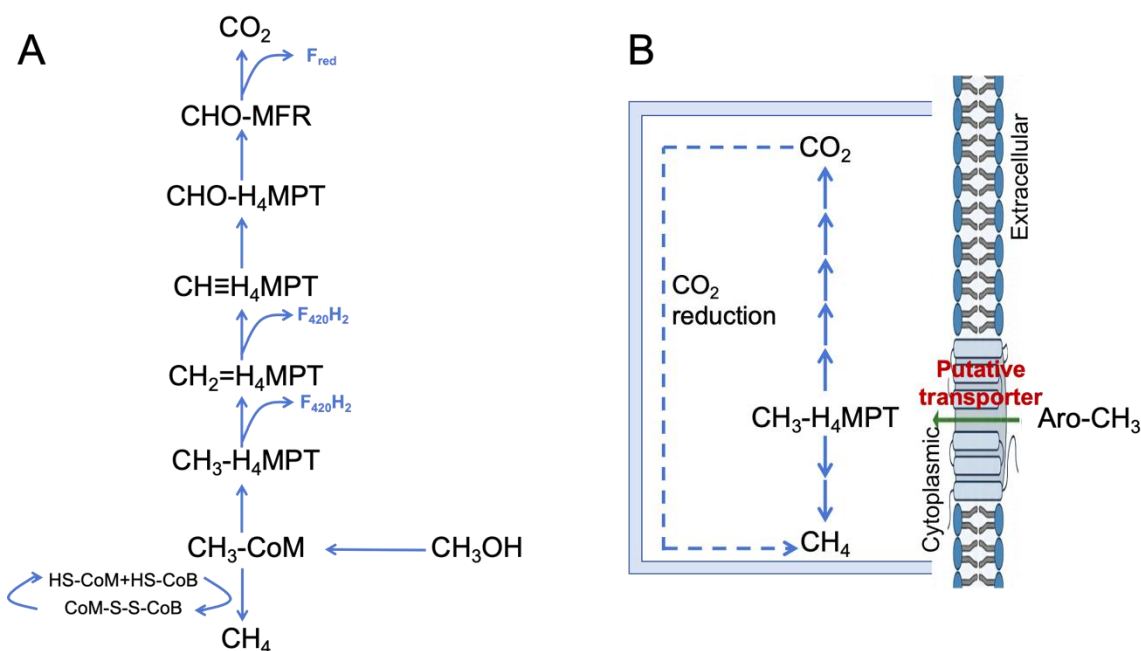


Figure 2-3. Distinct carbon flow patterns in classical methylotrophic and methoxydotrophic methanogenesis. **A.** In classical methylotrophic methanogenesis, simple methylated compounds enter the pathway via coenzyme M and are converted to methane. **B.** Methoxydotrophic methanogenesis in *Methermicoccus shengliensis*. Methoxylated aromatic compounds are imported into the cell via a putative membrane transporter. The methoxy-derived carbon is channeled into the methanogenic pathway through the C_1 carrier tetrahydromethanopterin ($\text{CH}_3\text{-H}_4\text{MPT}$). In parallel, cleavage of the substrate releases CO_2 , a fraction of which is subsequently reduced to methane, forming an internal carbon recycling loop. This dual carbon flux distinguishes

methoxydotrophic methanogenesis from classical pathways and highlights a unique route for methane production from structurally complex aromatic substrates.

2.6 Knowledge gaps and future perspectives

Despite substantial advances in elucidating methanogenic pathways and their underlying enzymatic mechanisms, a critical gap remains in our understanding of how substrates are transported into methanogenic cells. Current knowledge is largely focused on intracellular metabolic processes, whereas the initial step of substrate acquisition, which connects environmental availability with metabolic conversion, remains poorly characterized.

This limitation is particularly pronounced in recently discovered methanogenic pathways involving structurally complex substrates. Methoxydotrophic methanogenesis demonstrates that methanogens can utilize large, hydrophobic, and chemically complex compounds derived from lignin or coal. Such molecules are unlikely to enter cells via passive diffusion alone, yet the transport systems responsible for their uptake have not been identified.

Emerging genomic evidence suggests that substrate transport and metabolism may be functionally coupled, as genes encoding putative membrane transporters are frequently co-localized with metabolic gene clusters. This genomic organization implies a coordinated system linking substrate uptake with downstream metabolic processing. However, direct experimental validation of these transport systems is still lacking, and their physiological roles remain largely speculative.

In this context, *Methermicoccus shengliensis* provides a unique model organism to address this knowledge gap. As the only cultivated archaeon known to perform methoxydotrophic methanogenesis, it offers a tractable system to investigate the mechanisms underlying the uptake of complex organic substrates. Notably, a gene encoding a MatE family transporter is located adjacent to the methoxylated substrate utilization gene cluster in its genome, suggesting a potential role in substrate transport.

Elucidating the function of this transporter is therefore essential not only to establish a mechanistic link between extracellular substrate availability and intracellular methane production, but also to provide broader insights into the functional diversification of MatE transporters in archaea. In particular, understanding how these transporters contribute to the uptake of structurally diverse compounds may shed light on their role in ecological adaptation and metabolic flexibility. Such insights will ultimately help bridge the gap between environmental substrate complexity and methanogenic metabolism and lay the foundation for a systematic exploration of MatE transporter diversity, evolution, and function in archaea.

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Chapter 3

MatE transporter affects methane metabolism in *Methermicoccus shengliensis* and is modulated by methoxylated aromatic compounds

Short overview of chapter III

Chapter III focuses on the functional characterization of the MatE transporter in *Methermicoccus shengliensis*. Using molecular, structural, and genetic approaches, this chapter investigates the potential involvement of MatE and its associated Tlp protein in the uptake of methoxylated aromatic compounds and examines the consequences of *matE* deletion for growth and methane production. The findings provide experimental evidence linking an archaeal MatE transporter to substrate uptake and methanogenesis.

3.1 Abstract

Methoxylated aromatic compounds are abundant in subsurface ecosystems. Recently, it was discovered that *Methermicoccus shengliensis* can convert methoxylated aromatics to methane. Specifically, the MATE family transporters (MatE) and transduction-like protein (Tlp) were hypothesized to play a crucial role in substrate transport. However, their biological function and the transporting model remained unclear. To address this knowledge gap, we employed bacterial two-hybrid and structural model assays to investigate the interaction between Tlp and MatE. Our results revealed that Tlp senses 2-methoxybenzoate and interacts with MatE to facilitate substrate transport. Furthermore, we observed that the *matE* knock-out mutant significantly impaired the growth and methane production of *M. shengliensis* when using 2-methoxybenzoate as a substrate, highlighting the essential role of MatE in methoxydotrophic methanogenesis. Overall, our findings suggest that the MatE-Tlp system regulates substrate uptake and methane metabolism in *M. shengliensis*, providing new avenues for reducing global methane emissions caused by methanogens.

Keywords: Methanogens, Methane metabolism, 2-methoxybenzoate, MATE family transporter, Chemoreceptor.

3.2 Introduction

Methanogenic archaea are a crucial component of the global carbon cycle, producing approximately 1 billion metric tons of methane annually (Costa et al., 2014; Thauer et al., 2008). These microorganisms thrive on a limited range of substrates, deriving energy from methanogenic processes (Lyu et al., 2018). While most methanogens rely solely on H₂/CO₂ or formate for growth, members of the *Methanosarcina* genus exhibit greater metabolic diversity. For instance, certain species can utilize a broader range of substrates, including H₂/CO₂, simple carbon compounds (C1-C2, such as methanol, methylamines, dimethylamines, trimethylamines, methanethiol, and methyl-sulfides), and acetate (Nayak et al., 2019). Notably, *Methermicoccus shengliensis*, a thermophilic methanogen isolated from a deep subsurface environment (Cheng et al., 2007), has been found to directly convert a variety of methoxylated aromatic compounds (MACs) into methane (Mayumi et al., 2016). This unique ability sets it apart from other methanogens and highlights its potential role in the global carbon cycle.

Transcriptomic analysis indicated that two highly expressed genes, BP07_RS03225 (annotated as putative efflux transporter) and BP07_RS04910 (annotated as PAS domain S-box-containing protein), may be involved in substrate transport when *M. shengliensis* uses methoxybenzoate as substrate, by comparison with methanol. However, the biological function of these two genes has not yet been determined (Kurth et al., 2021).

To gain a deeper understanding of these two proteins, we conducted primary structural analysis. The protein encoded by BP07_RS03225 was found to belong to the MATE family of transporters (we named the MatE), consisting solely of the MatE domain. This domain functions as a transmembrane transporter, responsible for transporting substrates across the cell. In contrast, the protein encoded by BP07_RS04910 was classified as a methyl-accepting chemotaxis protein or transduction-like protein (Tlp). Tlp often contains several distinct regions, including transmembrane helices, Per-ARNT-Sim (PAS), PAS-associated C-terminal (PAC), Histidine kinases, Adenyl cyclases, Methyl accepting chemotaxis proteins (MCPs), and Phosphatases (HAMP) (Aravind et al., 1999; Williams et al., 1999), as well as a methyl-accepting chemotaxis-like (MA) domain. The PAS domain is capable of sensing environmental factors that cross the cell membrane and/or affect cell metabolism (Taylor et al., 1999), while the HAMP domain is frequently found in proteins associated with signal transduction in prokaryotic and lower eukaryotic organisms. The HAMP domain plays a critical role in transmitting conformational changes from ligand-binding domains to signaling domains (Aravind et al., 1999). The MA domain, present in chemotaxis sensory transducers, contributes to chemotactic behavior.

In this study, we employed bioinformatics analysis to predict that Tlp senses the substrate 2-methoxybenzoate, which is then transported into the cytoplasm by MatE. To validate this hypothesis, we conducted bacterial two-hybrid and structural model assays to elucidate the mechanism of the MatE-Tlp transport system. Furthermore, the poor growth and methane production of the *matE* knock-out mutant provided additional evidence for the critical role of MatE in methoxytrophic methanogenesis. Our comprehensive analysis, combining genetic, biochemical, and structural model approaches, revealed the intricate interactions between Tlp and MatE, as well as their impact on substrate uptake and subsequent methane emission behavior. This study provides the first analysis of the transport model of MatE-Tlp in *M. shengliensis*, shedding light on the methane emission reduction and methane production regulation on specific ecosystem.

3.3 Material and Methods

3.3.1 Microbial strains and culture conditions

The complete list of strains, primers and plasmids used in this study was shown in **Table 3-S1** and **Table 3-S2**, respectively. *Escherichia coli* DH5 α and BTH101 were cultured on LB agar plates or LB broth supplemented with ampicillin (100 $\mu\text{g}\cdot\text{mL}^{-1}$) or kanamycin (50 $\mu\text{g}\cdot\text{mL}^{-1}$) at 37 °C. *M. shengliensis* ZC-1 (DSM18856) was stored in our laboratory (Chengdu, China) and cultivated in modified DSM medium 1084 (Cheng et al., 2007). Sludge fluid was replaced by trace element solution (100 $\times$ trace element solution), as previously reported (Kurth et al., 2021). The medium was sparged with N₂: CO₂ in an 80:20 ratio before autoclaving. As substrate either 30 mM methanol or 30 mM 2-methoxybenzoate were used. The cultures were incubated at 65 °C. The growth was determined by measuring the optical density at 600 nm (OD₆₀₀) and the methane production at different times. The identity of the microorganism was checked by 16S rRNA gene sequencing of DNA from methanol and 2-methoxybenzoate-grown cells with primers show in **Table 3-S1**.

3.3.2 Determination of the minimal inhibitory concentration (MIC)

Liquid minimal inhibitory concentrations (MIC)s was determined as follows: saturated liquid cultures (OD₆₀₀ = 0.35 for *M. shengliensis*) were used to inoculate liquid media containing different concentrations of puromycin (0 - 5 $\mu\text{g}\cdot\text{mL}^{-1}$), neomycin (0 - 5 $\text{mg}\cdot\text{mL}^{-1}$), and nourseothricin (0 - 200 $\mu\text{g}\cdot\text{mL}^{-1}$) at a 1:100 final dilution of cells. Growth on liquid containing the different concentrations of antibiotics was monitored over time and compared with growth on media lacking antibiotics for MIC determination.

3.3.3 Total RNA extraction and quantitative real-time PCR (qRT-PCR)

M. shengliensis was cultivated in 120 mL serum vials containing 40 ml of the medium with 30 mM methanol, or 2-methoxybenzoate as the substrates. Cells in the mid-exponential growth phase were collected from all treatments for total RNA extraction using the RNeasy pure Cell/Bacteria Kit (TIANGEN, China). RNA purity and concentrations were assessed through gel electrophoresis and measured using a NanoDrop2000 spectrophotometer (Thermo Scientific, USA). Ribosomal RNA was removed from total RNA using the RiboMinus TM kit (Invitrogen, USA) (Zhang et al., 2024).

For the qRT-PCR analyses, total RNA was extracted following the method described above and treated with DNase I to eliminate DNA contamination. The RNA was then reverse transcribed into cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad, USA). Transcript levels were quantified using the SsoAdvanced™ Universal SYBR Green Supermix and a CFX96 Connect™ Real-Time PCR Detection System (Bio-Rad, USA). The PCR cycling conditions were as follows: initial denaturation at 95°C for 30 s,

followed by 40 cycles of 94°C for 15 s and 60°C for 30 s. After the main program, melt curve analysis was performed from 65 °C to 95 °C, with an increment of 0.5 °C and 0.5 s plate reading at each step. If necessary, sample DNA was diluted approximately 50-fold. Positive controls (using chromosomal DNA for qPCR) and negative controls (using RNA without reverse transcriptase) were included in the experiment and subjected to identical amplification conditions. The 16S rRNA gene served as an internal standard to normalize the results. Relative transcript levels of target genes were calculated using the Quantitation-Comparative CT($2^{-\Delta\Delta CT}$) method (Livak et al., 2001; Zhang et al., 2024).

3.3.4 Bacterial two-hybrid (bacterial two-hybrid) and β -galactosidase assay in 96-well arrays

The bacterial two-hybrid assays as described previously (Battesti et al., 2012). To study protein-protein interactions, the *tlp* gene was cloned into the pKNT25 vector, resulting in a fusion of the C-terminal catalytic domain of CyaA with the T25 fragment. The *matE* gene was cloned into the pUT18C vector, resulting in a fusion with the T18 catalytic domain fragment of CyaA at either the C-terminus or N-terminus. Cloning was performed using *E. coli* BTH101 competent cells. Co-transformed cells were plated on LB agar containing final concentrations of 100 μ g/ml ampicillin, 50 μ g/ml kanamycin, 0.5 mM IPTG and 50 μ g/ml X-gal (Zhang et al., 2024). The collected cells were cultivated overnight at 30°C. The pKNT25-zip and pUT18C-zip plasmids were used as positive controls, and the pKNT25/pUT18C (without insert) and pUT18C-zip/pKNT25-zip (fused with a leucine zipper protein) plasmids were used as negative controls, respectively. In the experimental group, the two fragments were fused into two interacting proteins. The bacterial two-hybrid assay primers and plasmids are listed in **Table 3-S1** and **Table 3-S2**, respectively.

For β -galactosidase assay was adapted to the 96-well array by modification of the Battesti & Bouveret's method (Battesti et al., 2012). Briefly, an overnight culture was 100-fold diluted with fresh LB liquid and grown at 37°C. When the culture reaches an OD₆₀₀ of 0.4-0.6, add IPTG to a final concentration of 0.5 mM. After inducing for 1-3 hours, 1 ml of cultures were used for β -galactosidase activity assay using the reported method following Battesti et al. The difference is that enzymatic reaction is carried out at 37 °C for 0 - 500 min with a measurement of OD₄₂₀ every 30 min in the microplate reader. The relative β -galactosidase activity in each sample was then calculated by simple excel file manipulation. The following calculation method is ((OD₄₂₀ at time t₂-OD₄₂₀ at time t₁) / t₂-t₁ (min)) / OD₆₀₀. The t₂ and t₁ time points are selected to be in the linear part of the kinetic.

3.3.5 *M. shengliensis* knock-out strain construction

The construction of *M. shengliensis* knock-out mutant strains was carried out in the following manner. Initially, the flanking regions of the selected open reading frames (ORFs) were amplified using PCR. A puromycin resistance cassette was then inserted in the middle of these regions. The entire element containing the cassette was then cloned into a pBluescript II SK plasmid using the Gibson assembly protocol (Gibson et al., 2009). The resulting plasmids were used to integrate the mutated alleles into the chromosomes of *M. shengliensis* through liposome-mediated transformation and allelic recombination. Notably, deletions of individual gene removed most of the coding sequence, resulting in an in-frame fusion peptide consisting of the first and last 10 amino acids, thus creating non-polar mutations. Specifically, the cassette lacks both a promoter or a terminator (Zhang et al., 2024). It contains translational stop codons in all reading frames preceding the 5'-end of the ribosome binding site (RBS) of the *pac* encoding sequence, and another RBS at the 3'-end to prevent translational coupling with the downstream *matE* gene. The primers and plasmids used to generate the mutants are listed in **Tables 3-S1** and **Tables 3-S2**, respectively.

3.3.6 Transformation methods

Escherichia coli strains were transformed by heat shock at 42 °C. For *M. shengliensis*, liposome-mediated transformation was used. Briefly, cells from log-phase cultures ($OD_{600}=0.35$) were collected by centrifugation and resuspended in 0.85 M sucrose to a density of about 10^9 cells per millilitre. Plasmid DNA-liposome complexes were formed by mixing 15 µL of DOTAP (Boehringer Mannheim) in 100 µL of 20 mM Hepes (pH 7.6) with 1.5 µg of plasmid DNA in 50 µL of 20 mM Hepes (pH 7.4). This was followed by a 30-minute incubation at 65 °C. A 1.0-mL portion of the resuspended cells was added to the plasmid DNA-liposome suspension and incubated for 6 hours at room temperature. With these cells and DNA concentrations, the maximum transformation frequency of *M. shengliensis* was achieved with 15 µL of DOTAP reagent. Following all methods, cells were transferred to 10 mL of broth medium after transformation, incubated at 45 °C for 16 hours, and then plated on a medium with 2.5 µg/mL puromycin. All *M. shengliensis* transformants were single colony purified on solid media containing puromycin before undergoing PCR verification and phenotypic analysis.

3.3.7 AlphaFold2 prediction and docking analysis

The amino acid sequences of MatE and Tlp were retrieved from the genome of *Methermicoccus shengliensis* DSM 18856. Three-dimensional protein structures were predicted using AlphaFold2 (Jumper et al., 2021), and model confidence was evaluated

based on predicted local distance difference test (pLDDT) scores. Only regions with high confidence (pLDDT > 70) were retained for subsequent analyses. Prior to docking, protein structures were prepared using UCSF Chimera (version 1.12) (Pettersen et al., 2004), including removal of low-confidence regions where necessary, addition of hydrogen atoms, and assignment of appropriate atom types and charges. The ligand 2-methoxybenzoate was obtained from the PubChem database and prepared by geometry optimization and assignment of Gasteiger charges.

Molecular docking was performed using AutoDock Vina (version 1.1.2) (Trott et al., 2010). In the absence of experimentally validated binding sites, a two-step docking strategy was employed. First, blind docking was conducted to identify potential ligand-binding regions across the entire protein surface. Based on these results and structural features of MATE transporters, a focused docking approach was subsequently applied by defining a grid box encompassing the central cavity within the transmembrane domain, which is known to be involved in substrate recognition and transport. Docking parameters were set as follows: the number of binding modes was set to 8, exhaustiveness to 8, and the maximum energy difference to 3 kcal·mol⁻¹. The best-ranked binding poses were selected based on binding affinity and structural plausibility. All docking results were visualized and analyzed using PyMOL (Schrodinger, LLC).

3.3.8 Genome analysis

The whole genome of *M. shengliensis* was downloaded from the NCBI database, and an operon analysis was conducted (Altschul et al., 1997). All the candidates were then reexamined for their domain organization using the SMART database (http://smart.embl-heidelberg.de/smart/set_mode.cgi?GENOMIC=1) (Letunic et al., 2021). The locations of domains in Tlp and MatE were analyzed using the TMHMM and TMPred programs (Hargrave et al., 1993; Krogh et al., 2001).

3.3.9 Phylogenetic analysis

The MatE and Tlp sequence from *M. shengliensis* and homologous MatE/Tlp sequences from publicly available archaea species were aligned using MEGA 11 (**Table 3-S1 and 3-S2**) (Stecher et al., 2020; Tamura et al., 2021). A maximum-likelihood phylogenetic tree based on the alignments was constructed by FastTree v2.0 under the JC model, and bootstrap analysis was applied with 1,000 replications (Price et al., 2010). The initial tree for the heuristic search was automatically generated.

3.3.10 Growth curves and chemical analyses

M. shengliensis, grown in the presence of either 2-methoxybenzoate or methanol, were maintained at 65 °C at all times. After adapting to the respective substrate, the culture is further passaged in a medium containing antibiotic, typically for 3 generations, with an inoculation amount of 2%. The cultures were then incubated in quadruplicates at a steady temperature of 65 °C. Growth was measured every 12 or 24 hours by measuring the optical density at 600 nm (OD₆₀₀) with a Spectronic 21 spectrophotometer (Milton Roy, USA).

Gas samples (200 µL) were collected from the headspace of vials or serum bottles at various time points using a pressure-lock syringe (Vici, Schenkon, Switzerland). Methane (CH₄) concentrations were analyzed with an Agilent GC7820A gas chromatography system (Agilent, USA) equipped with a Porapak Q column and a thermal conductivity detector. The column, oven, and detector temperatures were set to 65 °C, 120 °C, and 130 °C, respectively. Argon served as the carrier gas at a flow rate of 27 mL min⁻¹. The gas pressure in the headspace of vials or serum bottles was measured at room temperature using a barometer (Ashcroft, Stratford, CT). The total amount of gas products was calculated based on Avogadro's law, following calibration with a standard gas mixture (composition: N₂: CH₄: C₂H₆: C₃H₈: 2-C₄H₁₀: 2-C₅H₁₂ = 61.41% : 14.24% : 8.21% : 10.02% : 3.80% : 2.32%). The concentrations of methane were determined using an external calibration curve (Zhang et al., 2024).

3.3.11 Statistics and reproducibility

Every experiment was conducted with a minimum of three independent biological replicates. Data are presented as the mean $\pm$ standard deviation (SD). Data points in figures represent biological replicates. Each graph displays individual data points. Statistical analyses (mean and SD) were performed using GraphPad Prism 10 software. Detailed information on all reagents and resources can be found in “Material and methods” section.

3.4 Results and Discussion

3.4.1 Cell growth and methane production of *M. shengliensis* on different substrates

The growth curves of *M. shengliensis* varied significantly depending on the substrate used. Specifically, the growth rate of methanol was significantly higher than that of 2-methoxybenzoate. When methanol was used as the substrate, *M. shengliensis* reached the logarithmic growth phase within four days, whereas it took five days to reach this phase when 2-methoxybenzoate was used (**Figure 3-S1-A**). Furthermore, *M. shengliensis* produced more methane from methanol than from 2-methoxybenzoate. After seven days of cultivation, approximately 13 mmol of methanol and 10 mmol of 2-methoxybenzoate

were consumed for methane production, respectively ((**Figure 3-S1-B**). These results provide strong evidence that *M. shengliensis* has a preference for methanol as a substrate for both growth and methane production.

3.4.2 Diversity of phylogenetic MatE and Tlp

Previous studies have shown that MatE and Tlp are highly expressed during methoxydotrophic methanogenesis (Kurth et al., 2021). Our quantitative real-time PCR (qRT-PCR) analysis revealed that the relative transcription levels of both genes were significantly upregulated (**Figure 3-S2**). To further explore the distribution of MatE and Tlp in archaea, we constructed a phylogenetic tree. Our results showed that MatE and Tlp are widely distributed across archaea (**Figure 3-S3 and 3-S4**). Notably, while many archaea possess MatE and Tlp, only *M. shengliensis* is able of utilizing methoxylated aromatic compounds to produce methane, aside from the non-methanogenic archaeon *Archaeoglobus fulgidus* (**Table 3-S3 and 3-S4**).

3.4.3 MatE interacts directly with the Tlp

M. shengliensis has been shown to utilize methoxylated aromatics, such as 2-methoxybenzoate for growth (**Figure 3-S1**). Our primary structural model assay revealed that Tlp functions as a substrate signal sensing protein, transporting the substrate to MatE, which then moves the compound into the cytoplasm (**Figure 3-1A and 3-1B**). In other words, Tlp senses the signal to the transporter by interacting with MatE. To confirm the protein interaction, we constructed a bacterial two-hybrid system for Tlp and MatE.

Tlp consists of a ligand-binding domain (LBD), transmembrane (TM) helices, and a cytoplasmic signaling domain (SD) that interacts with downstream regulatory proteins (**Figure 3-1B**) (Bi et al., 2015; Wadhams et al., 2004). This enables Tlp to directly recognize ligands and mediate cellular responses (Kossmann et al., 1988; Machuca et al., 2016). To further understand how Tlp interacts with MatE, we used the bacterial two-hybrid system to confirm the interactions between LBD, TM, SD, and MatE, respectively. The interaction results showed that Tlp and HAMP interacted with MatE, and the blue spots in the experimental group were consistent with the control group (**Figure 3-1C and 3-1D**). Additionally, the activity of the adenylate cyclase was restored when the fragments were brought into proximity by the interaction of the two chimeric proteins, indicating a direct interaction between Tlp and MatE (**Figure 3-S5**).

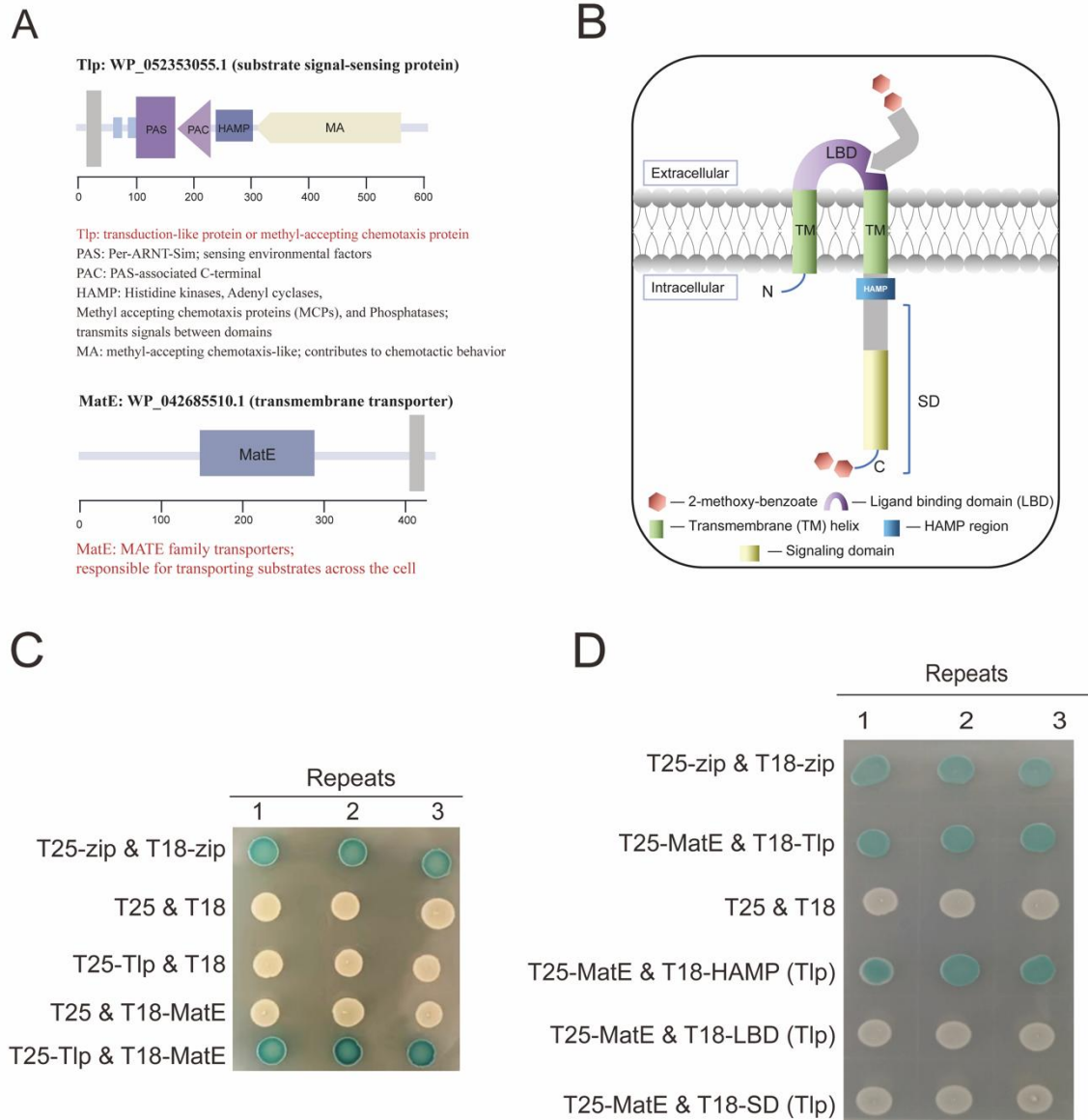


Figure 3-1. Tlp interacts with MatE and promotes its sensing to the substrate. **A.** The domain structures of Tlp and MatE were analyzed using the Pfam and SMART programs. **B.** The locations of domains in Tlp and MatE were analyzed using the TMHMM and TMpred programs. **C.** Bacterial two-hybrid assay analysis was conducted to examine the interaction between Tlp and MatE *in vivo*. **D.** To investigate the interaction between MatE and Tlp's domains, a bacterial two-hybrid assay was conducted. This analysis aimed to examine the *in vivo* interactions between MatE and the HAMP, LBD, and SD domains of Tlp.

To investigate how Tlp and MatE transport 2-methoxybenzoate into *M. shengliensis*, we designed the structure of Tlp and MatE using AlphaFold2. MatE mainly consists of

two groups of α -helix, each with six α -helix (**Figure 3-2A**). A substrate tunnel formed between this two group α -helix. Tlp consists of a ligand-binding domain, transmembrane helices, and a cytoplasmic signaling domain, which were predicted with high confidence (pLDDT scores >90) (**Figure 3-2B**). Molecular docking analysis showed that MatE interacts with the HAMP domain of Tlp (**Figure 3-2C**), which matches with protein interaction assays (**Figure 3-1D**). Additionally, in membrane-associated proteins, HAMP domains usually lie near the cytoplasmic side of the membrane, where they convert transmembrane and intracellular sensory inputs into output response signals (Parkinson, 2010). This suggests that the HAMP domain is crucial in the substrate transport process. The molecular docking result also showed that 2-methoxybenzoate has a high affinity (described by a lower binding energy of $-4.8 \text{ kcal mol}^{-1}$) for MatE. 2-methoxybenzoate binds to the substrate tunnel by hydrogen bonds with residues Ser³⁰² and Arg²⁷⁹ (**Figure 3-2D**). Furthermore, another methoxylated aromatic, 3,4,5-trimethoxybenzoate, docked also well with MatE (**Figure 3-S6**), while methanol did not interact with MatE, indicating that MatE and Tlp prefer to interact with methoxylated aromatics.

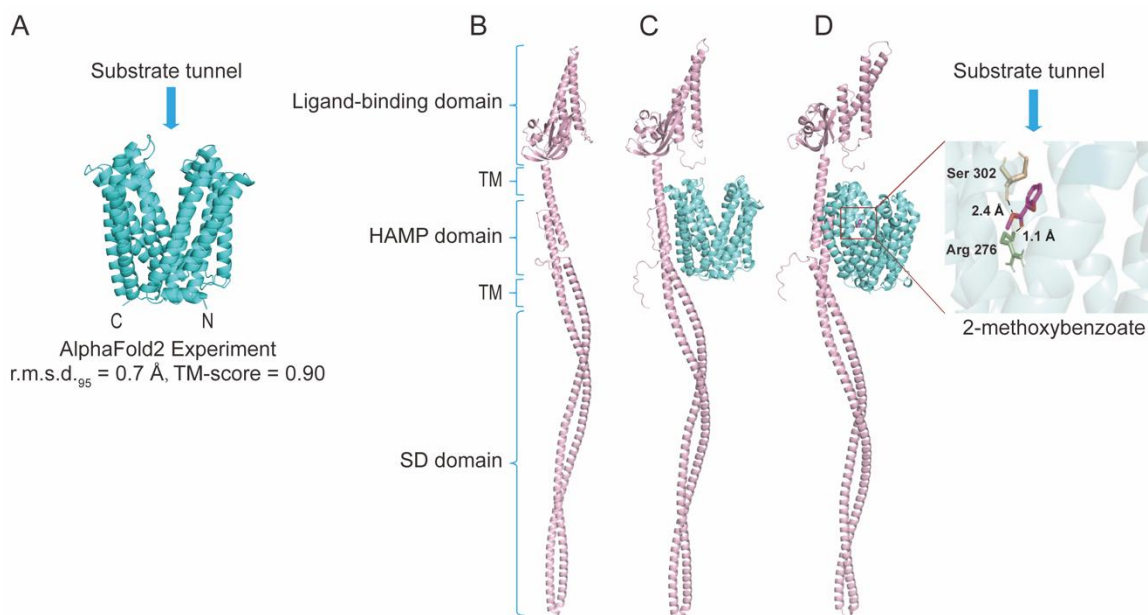


Figure 3-2. Ligand-MatE interactions in the middle membrane region of the transportation cavity. **A.** An overview of the MatE model structure (cyan). **B.** An overview of the Tlp model structure (purple). **C.** The best interaction modes of MatE (cyan) and Tlp (purple) created using AutoDock Vina. **D.** Molecular docking methods were used to identify 2-methoxybenzoate (red) sites on the surface and in the centre of the protein cavity. Hydrogen bonds are shown as black dashed lines.

Among the thirty MatE homologous proteins in archaea, we found that residue Arg²⁷⁹ was highly conserved (**Figure 3-S7**), suggesting that the MatE-Tlp system might function for all methylated aromatics. In summary, the LBD domain binds an extracellular substrate and transfers it to the HAMP domain, and then the HAMP domain interacts with MatE to transport the substrate into the cell.

3.4.4 The *matE* gene affects growth and methane production when *M. shengliensis* grown on 2-methoxybenzoate

The lack of selection markers has limited the development of genetic techniques in methanogens. To address this, we tested the effectiveness of puromycin, nourseothricin, and neomycin on *M. shengliensis* by determining their minimum inhibitory concentration (MIC) in liquid media at 45°C. Our results showed that growth of *M. shengliensis* was completely inhibited by 2.5 µg/mL of puromycin, 100 µg/mL of nourseothricin, and 1 mg/mL of neomycin, respectively (**Figure 3-3A** and **Figure 3-S8**). We also found that higher concentrations of puromycin were required to inhibit growth at 65°C, and the inhibitory effect persisted for 10 days (**Figure 3-3B**).

To explore the utility of the *pac* gene (encoding puromycin N-acetyltransferase) as a selectable marker for creating chromosomal deletions in *M. shengliensis*, we designed a plasmid to replace the *matE* gene with the *pac* marker (**Figure 3-3C**, **Figure 3-S9**). We expected that incorporation of the *pac* marker into the chromosome would render the cells resistant to puromycin (**Figure 3-3D**). To verify the integration of the *pac* marker, we designed primers outside the homologous recombinant plasmid region and on the *pac* cassette for PCR and sequencing verification (**Figure 3-S10**). Our results showed that a $\Delta matE$ mutant was successfully obtained in the transformants (**Figure 3-S11**).

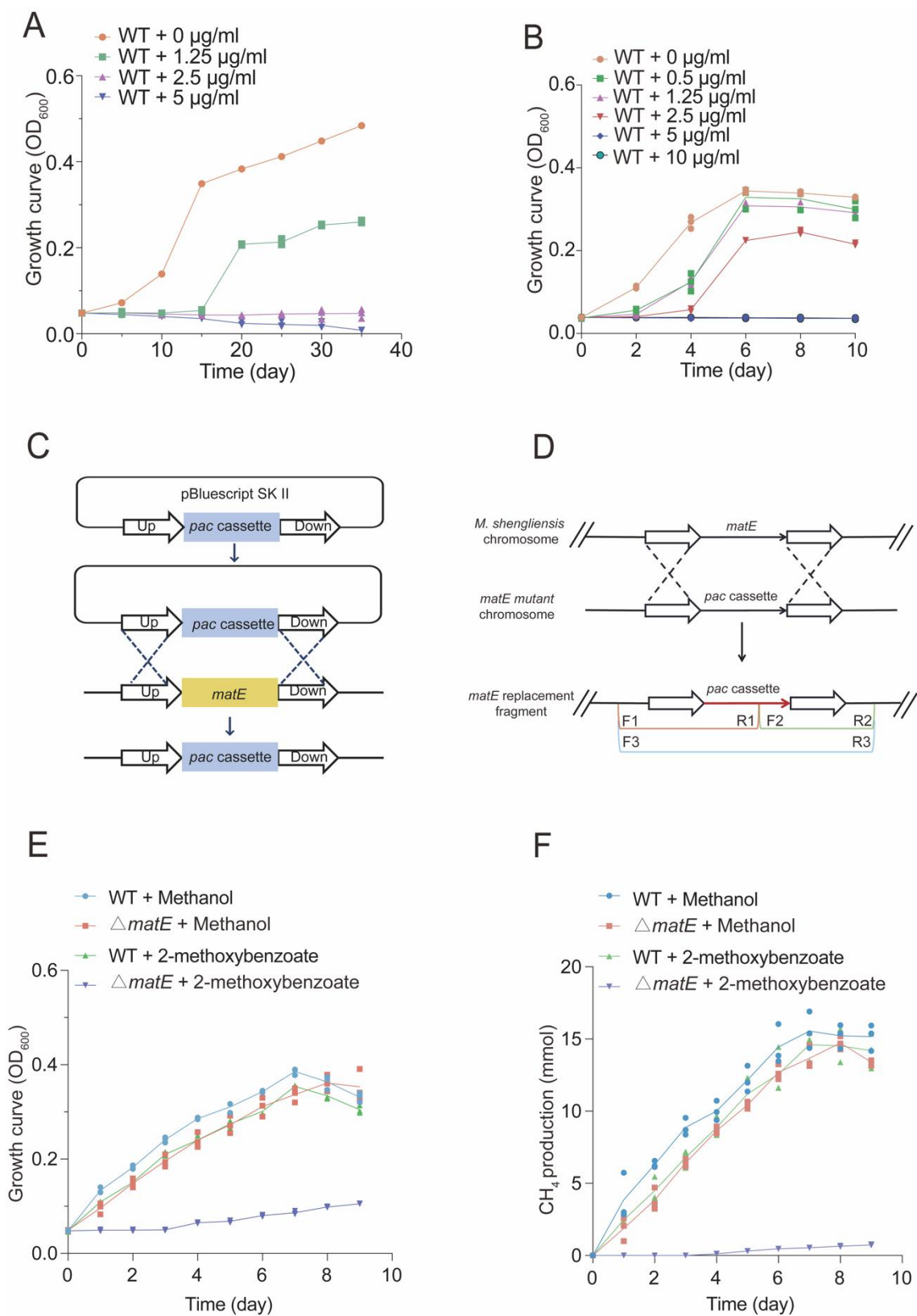


Figure 3-3. The *matE* gene influences growth and methane production in *M. shengliensis* when grown under 2-methoxybenzoate. **A.** Growth curves of *M. shengliensis* under varying concentrations of puromycin (0 - 5 $\mu\text{g}\cdot\text{mL}^{-1}$) in methanol under 45 °C. **B.** Growth curves of *M. shengliensis* under varying concentrations of puromycin (0 - 10 $\mu\text{g}\cdot\text{mL}^{-1}$) in methanol under 65 °C. **C.** Schematic representation of the marker mutagenesis technique for the *M. shengliensis* strain. **D.** A schematic of the generation of the *M. shengliensis* ΔmatE mutant. A linear DNA fragment containing *pac*, flanked by 700 bp regions homologous to the upstream and downstream regions of *matE*, was transformed into *M. shengliensis*. Incorporation of the *pac*-containing DNA at the *matE* locus gives rise to mutants that are resistant to puromycin. Primers used to verify ΔmatE mutants are represented by the F-R in the schematic. **E.** Growth curves for the wild-type *M. shengliensis* and the ΔmatE mutant when grown with methanol and 2-methoxybenzoate. **F.** Accumulation of methane in the headspace of bottles with wild-type *M. shengliensis* and the ΔmatE mutant when grown with methanol and 2-methoxybenzoate over an incubation period of 10 days. All data represent the mean of $n=3$ biologically independent samples (**A, B, E, F**).

To assess the genotype of ΔmatE mutant, we observed the growth and methane production of the ΔmatE mutant under different substrate conditions. Our results showed that the deletion of the *matE* gene greatly inhibited the growth of *M. shengliensis* when 2-methoxybenzoate was used (**Figure 3-3E**). Additionally, methane production in the ΔmatE mutant decreased about 95% compared to the wild-type, suggesting that the deletion of *matE* negatively impacts methanogenesis (**Figure 3-3F**). Our results also showed that the deletion of *matE* affected *M. shengliensis* growth and methane accumulation when 2-methoxybenzoate was used as the substrate, but not when methanol was used as the substrate.

In summary, our results suggest that MatE is important in methoxydotrophic methanogenesis in *M. shengliensis*, facilitating the entry of substrates into the cells from the extracellular environment. Unfortunately, we were unable to successfully establish a complementation mutant of *M. shengliensis*, highlighting the need for the development of genetic tools. Therefore, a better understanding of substrate transporters and methane metabolism in *M. shengliensis* is essential, and the development of more sophisticated genetic tools is urgently required to fill knowledge gaps and facilitate further research.

3.5 Supplementary files

Table 3-S1. List of the primer sequences used in this study.

Name	Primer Sequence (5' → 3')
109_F	ACKGCTCAGTAACACGT
915_R	GTGCTCCCCCGCCAATTCCT
F ₁	GACAACGTGAAGCCGAAGGGAATA
R ₁	AGATTTTAGCAGGTAAGATAGGGTCTC
F ₂	GAGACCCTATCTTACCTGCTAAAATCT
R ₂	GCGTTAAAGCTTTGATGTACTGCAAGT
F ₃	GACAACGTGAAGCCGAAGGGAATA
R ₃	GCGTTAAAGCTTTGATGTACTGCAAGT
pBluescript II SK_F	CAATTCGCCCTATAGTGAGTCG
pBluescript II SK_R	TTTAGTGAGGGTTAATTCGAGCT
<i>tlp</i> _F	GCGACAGGAGAGACAATACCGATTCTGTCT
<i>tlp</i> _R	AGCTCTCTGATGCCACTGCTGAGC
<i>matE</i> _F	AGATAGGCATTCCCGCATCAGTG
<i>matE</i> _R	GGCTGTCTTGCTCCAAGGTTCTG
16S_F	AACGATGCTCGCTAGGTGTCA
16S_R	AACTACGGTTGGGTTTAGGAGATTA
q16S_F	CAGGGGTAGGGGTGAAATCTTGTGA
q16S_R	GACACCTAGCGAGCATCGTTTACGGC
pUT18C-F	TCGAATTCAGCCGCCAGCGAGGCCA
pUT18C-R	TGGCGTAATCATGGTCATAGCTGTTTCCT
pKNT25-F	CCGAGCTCGAATTCAATGACCATGCAG
pKNT25-R	TACCCGGGGATCCTCTAGAGTCGA
T18_ <i>matE</i> _F	AGGAAACAGCTATGACCATGATTACGCCAATGATGATCAGCAACC TCGTTTTCACT
T18_ <i>matE</i> _R	TGGCCTCGCTGGCGGCTGAATTCGACCGTAACTCTCTTTCAAGCCT TCTGATGC
T25_ <i>tlp</i> _F	CAGGTCGACTCTAGAGGATCCCCGGGTAATGCCAGTAAATGGTTAT TCGAGCACAAC
T25_ <i>tlp</i> _R	CTGCATGGTCATTGAATTCGAGCTCGGCTCAGCTCGACTTTGCTTG GCCT
T18_HAMP_F	TGGTAAGAAAAATCGCAATGAAGA
T18_HAMP_R	AAGTATTCATTACGGATAATAC
T18_LBD_F	CGTAAAATGTGTAGAATCAGG
T18_LBD_R	CGAAATCTACGTTGTAGCATCTGGA
T18_SD_F	TAACAGTAGAAACGTAGATGGTGAG
T18_SD_R	GGACGAATTAGAAGAATTAACCTTCAAAATACGGATT

Table 3-S2. List of the microorganism strains and plasmids used in this study.

Plasmid/ strains	Relevant Genotype or description	Reference and Source
pMEV4	Source of the <i>pac</i> gene, Amp ^r .	(Akinyemi et al., 2021)
pBluescript II SK	pBluescript II SK (–) Multiple Cloning Site Region (sequence shown 598–826), Amp ^r .	TIANGEN, China
pKNT25-zip	This plasmid is T25 protein fusion of C-terminal zip.	TIANGEN, China
pUT18C-zip	This plasmid is T18 protein fusion of C-terminal zip.	TIANGEN, China
pKNT25	This plasmid is used to express T25 fused protein in <i>E.coli</i> ; Km ^r .	TIANGEN, China
pUT18C	This plasmid is used to express T18 fused protein in <i>E.coli</i> ; Amp ^r .	TIANGEN, China
pCH011	pUT18C containing the <i>matE</i> ; Amp ^r .	In this study
pCH012	pKNT25 containing the <i>tlp</i> , Km ^r .	In this study
pCH013	<i>matE</i> gene deleted vector for constitutive expression of genes by chromosomal integration, Pur ^r .	In this study
DH5α	supE44 DlacU169 (F80lacZDM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1	TIANGEN, China
BTH101	F0 cya-99 araD139 galE15 galK16 rpsL1 (StrR) hsdR2 mcrA1 mcrB1 relA1	TIANGEN, China
<i>M. shengliensis</i> DSM18856	Wild type strain	DSMZ, Germany
mMS01	<i>matE</i> gene deleted in wild-type (<i>matE::pur^r</i>). Pur ^r	In this study

Note: Amp^r, Km^r, and Pur^r indicate resistance to Ampicillin, Kanamycin, and Puromycin, respectively.

Table 3-S3. Summary of MatE homologs in archaea.

Strains	Protein ID	Length (aa)	Substrates	O-demethylation/methyl transferase system	References
<i>Methermicoccus shengliensis</i>	WP_042685510.1	451	MeOH, MA, MACs	BP07_RS03260, BP07_03255, BP07_03250, BP07_RS03245, BP07_RS03240, BP07_RS03235, BP07_RS03225, BP07_RS03220	(Kurth et al., 2021)
<i>Archaeoglobus fulgidus</i>	WP_010878968.1	459	CO, sulfate, lactate, pyruvate, formate, amino acids, MACs	AF_0006, AF_0007, AF_0008, AF_0009, AF_0010, AF_00011, AF_0012, AF_0013	(Welte et al., 2021)
<i>Archaeoglobus sulfaticallidus</i>	WP_015590434.1	457	H ₂ /CO ₂ (lithoautotrophically), sulfate, sulfite, thiosulfate	NA	(Steinsbu et al., 2010)
<i>Archaeoglobus sp.</i>	WP_290725701.1	452	H ₂ /CO ₂ /HC O ₃ ⁻ , sulfate, sulfite, thiosulfate	NA	(Slobodkina et al., 2021)
<i>Archaeoglobus i archaeon</i>	NOY11224.1	454	MA	NA	(Kashefi et al., 2002)
<i>Geoglobus ahangari</i>	WP_048094251.1	454	H ₂ (autotrophically)	NA	(Kashefi et al., 2002)
<i>Ferroglobus placidus</i>	WP_012966375.1	447	MACs	NA	(Schmid et al., 2015)
<i>Geoglobus acetivorans</i>	MBE8538645.1	453	CO ₂ (lithoautotrophically)	NA	(Mardanov et al., 2015)
<i>Thermoplasma matalae archaeon</i>	HHF58642.1	463	methyl compounds	NA	(Zhou et al., 2020)
<i>Thermococcus pacificus</i>	WP_088854540.1	458	sulfur	NA	(Miroshnichenko et al., 1998)
<i>Thermococcus sibiricus</i>	WP_012766284.1	458	d-(+)-glucose, starch, pyruvate	NA	(Park et al., 2023)
<i>Thermococcus sp.</i>	WP_297189308.1	458	yeast extract, tryptone, peptone	NA	(Yang et al., 2023)
<i>Pyrococcus furiosus</i>	WP_011011833.1	461	NA	NA	NA
<i>Thermoplasma</i>	MCD644845	459	NA	NA	NA

Functional characterization of a MatE transporter in *Methermicoccus shengliensis* and evolutionary diversity of archaeal MatE transporters

<i>mata archaeon</i>	1.1				
<i>Thermococci archaeon</i>	RLF81964.1	464	NA	NA	NA
<i>Thermococcus sp. 101 C5</i>	WP_371716967.1	458	NA	NA	NA
<i>Thermococcus sp. PK</i>	WP_042699710.1	460	NA	NA	NA
<i>Thermococcus</i>	WP_015849995.1	459	NA	NA	NA
<i>Archaeoglobaceae archaeon</i>	MCS7129840.1	458	xyloglucan	NA	(Buessecker et al., 2023)
<i>Ferroglobus sp.</i>	WP_290902083.1	431	NA	NA	NA
<i>Pyrococcus kukulkanii</i>	WP_372915409.1	455	yeast extract, peptone, tryptone	NA	(Callac et al., 2016)
<i>Archaeoglobales archaeon</i>	MDI9642395.1	444	xyloglucan	NA	(Buessecker et al., 2023)
<i>Methanobacteriota archaeon</i>	MEA1993320.1	455	NA	NA	NA
<i>Thermosiphon atlanticus</i>	WP_073073034.1	454	sea salts	NA	(Urios et al., 2004)
<i>Thermotoga sp. Mc24</i>	KHC92383.1	456	NA	NA	NA
<i>Methanophagaes archaeon</i>	MBE0515813.1	469	NA	NA	NA
<i>Methanofastidiosum sp.</i>	NPV50761.1	464	NA	NA	NA
<i>Methanofolliis sp.</i>	MDD3621053.1	471	NA	NA	NA
<i>Methanofastidiosum sp.</i>	MFA7690824.1	465	NA	NA	NA
<i>Methanofolliis sp. W23</i>	WP_209631151.1	471	NA	NA	NA

Notes: MeOH: methanol; MA: methylamines; MACs: Methoxylated aromatic compounds; NA: no found that in the literature.

Table 3-S4. Summary of Tlp homologs in archaea.

Strains	Protein ID	Length (aa)	Substrates	O-demethylation/methyltransferase system	References
<i>Methermicoccus shengliensis</i>	WP_084174117.1	339	MeOH, MA, MACs	BP07_RS03260, BP07_03255, BP07_03250, BP07_RS03245, BP07_RS03240, BP07_RS03235, BP07_RS03225, BP07_RS03220	(Kurth et al., 2021)
<i>Archaeoglobus fulgidus</i>	WP_010878545.1	834	CO, sulfate, lactate, pyruvate, formate, amino acids, MACs	AF_0006, AF_0007, AF_0008, AF_0009, AF_0010, AF_00011, AF_0012, AF_0013	(Welte et al., 2021)
<i>Methanobrevibacter sp. ZRKC2</i>	WYM77517.1	323	NA	NA	NA
<i>Methanobrevibacter sp. T82-4</i>	KXS42768.1	333	NA	NA	NA
<i>Methanohalophilus</i>	WP_096711806.1	328	MA	NA	(Guan et al., 2019)
<i>Methanohalophilus sp. T328-1</i>	KXS45615.1	336	NA	NA	NA
<i>Methanohalophilus sp.</i>	RSD33148.1	319	NA	NA	NA
<i>Promethearchaeia archaeon</i>	HMF31413.1	680	amino acids, peptides, yeast extract, H ₂ /formate-utilizing	NA	(Imachi et al., 2024)
<i>Methanoregula sp.</i>	MFA4876872.1	1921	NA	NA	NA
<i>Methanofollis liminatans DSM 4140</i>	EJG07942.1	740	NA	NA	NA
<i>Methanocaldococcus chunghsingensis</i>	WP_211531330.1	2069	formate, H ₂ /CO ₂	NA	(Lai et al., 2004)
<i>Haloarcula marismortui ATCC 43049</i>	QCP91753.1	522	NA	NA	NA
<i>Haloarcula marismortui</i>	WP_332379637.1	571	yeast extract, glucose, tryptone	NA	(Syutkin et al., 2019)

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<i>Salinigranum marinum</i>	WP_318567787.1	1013	maltose, sucrose, lactose, starch	NA	(Cheng et al., 2023)
<i>Theionarchaea archaeon</i>	MBU7019225.1	1116	detrital proteins, hemicellulose, ethanol, CO ₂	NA	(Cai et al., 2022)
<i>Thermoplasma archaeon</i>	KAA0000649.1	474	peptide/amino acid	NA	(Diamond et al., 2022)
<i>Methanoregula sp.</i>	MFA4825575.1	1367	NA	NA	NA
<i>Theionarchaea archaeon</i>	MBU7018316.1	796	detrital proteins, hemicellulose, ethanol, CO ₂	NA	(Cai et al., 2022)
<i>Archaeoglobus sp.</i>	MBO8183590.1	1082	H ₂ /CO ₂ /HCO ₃ ⁻ , sulfate, sulfite, thiosulfate	NA	(Slobodkina et al., 2021)
<i>Methanoculleus taiwanensis</i>	WP_128692812.1	590	formate, H ₂ /CO ₂	NA	(Weng et al., 2015)
<i>Methanobacteriota archaeon</i>	MBM4241490.1	824	NA	NA	NA
<i>Methanoregulae archaeon</i>	HHH02989.1	940	NA	NA	NA
<i>Archaeoglobus veneficus SNP6</i>	AEA47185.1	741	NA	NA	NA
<i>Methanoregulae archaeon</i>	HOV67018.1	866	NA	NA	NA
<i>Archaeoglobus sulfaticallidus PM70-1</i>	AGK62165.1	849	H ₂ /CO ₂ , thiosulfate	NA	(Stokke et al., 2013)
<i>Archaeoglobus sulfaticallidus</i>	WP_081623024.1	850	NA	NA	NA
<i>Methanoregulae archaeon</i>	HOT95202.1	945	NA	NA	NA
<i>Methanolinea mesophila</i>	WP_209672934.1	956	H ₂ /CO ₂ , formate	NA	(Sakai et al., 2012)
<i>Methanoculleus frigidifontis</i>	WP_301662866.1	1442	H ₂ /CO ₂ , formate	NA	(Lai et al., 2024)
<i>Methanolinea sp.</i>	MDI6899769.1	708	NA	NA	NA

Notes: MeOH: methanol; MA: methylamines; MACs: Methoxylated aromatic compounds; NA: no found that in the literature.

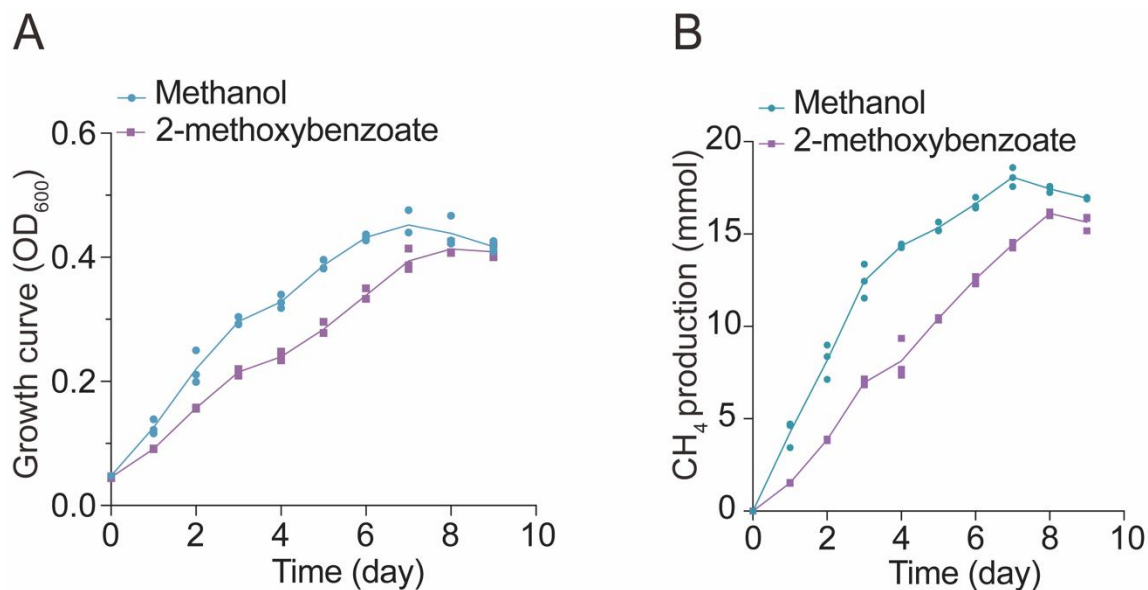


Figure 3-S1. Growth and methane production of *M. shengliensis* across different substrates. **A.** Growth curves of *M. shengliensis* under two distinct substrate regimes. **B.** Accumulation of methane in the headspace of bottles for different substrate treatments over a 10-day incubation period. All data represent the mean of n=3 biologically independent samples (**A, B**).

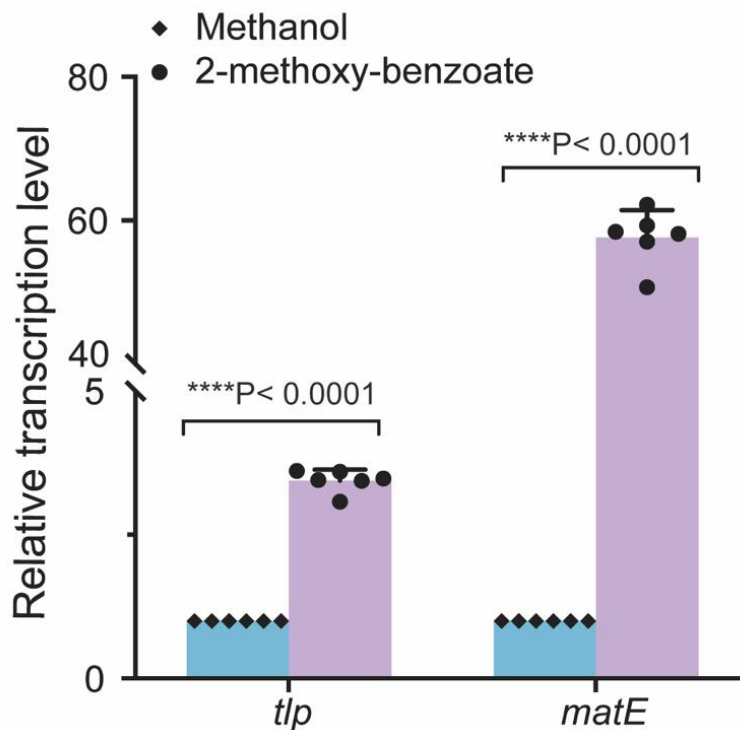


Figure 3-S2. Comparison of gene transcription during growth on methanol and 2-methoxybenzoate substrates. Transcription levels of *matE* and *tlp* were determined by qRT-PCR using 2-methoxybenzoate as substrate relative to methanol. All data represent the mean of $n=6$ biologically independent samples and error bars show standard deviation; Asterisks indicate statistically significant differences between the 2-methoxybenzoate and the methanol control ($****p < 0.0001$).



Figure 3-S3. Phylogenetic tree of MatE. A phylogenetic tree based on the full-length amino acid sequences of selected MatE proteins was constructed using the Maximum likelihood analysis method with 1000 bootstrap replications in MEGA 11 software. Note that the red pentagram indicates that archaea can use MACs to produce methane metabolism, and species that have been reported in the literature. The gene clusters depicted here encode proteins involved in O-demethoxylation/methyltransferase system (BP07_RS03260-BP07_RS03220; AF_0006-AF_0013). MeOH: methanol; MA: methylamines; MACs: Methoxylated aromatic compounds.

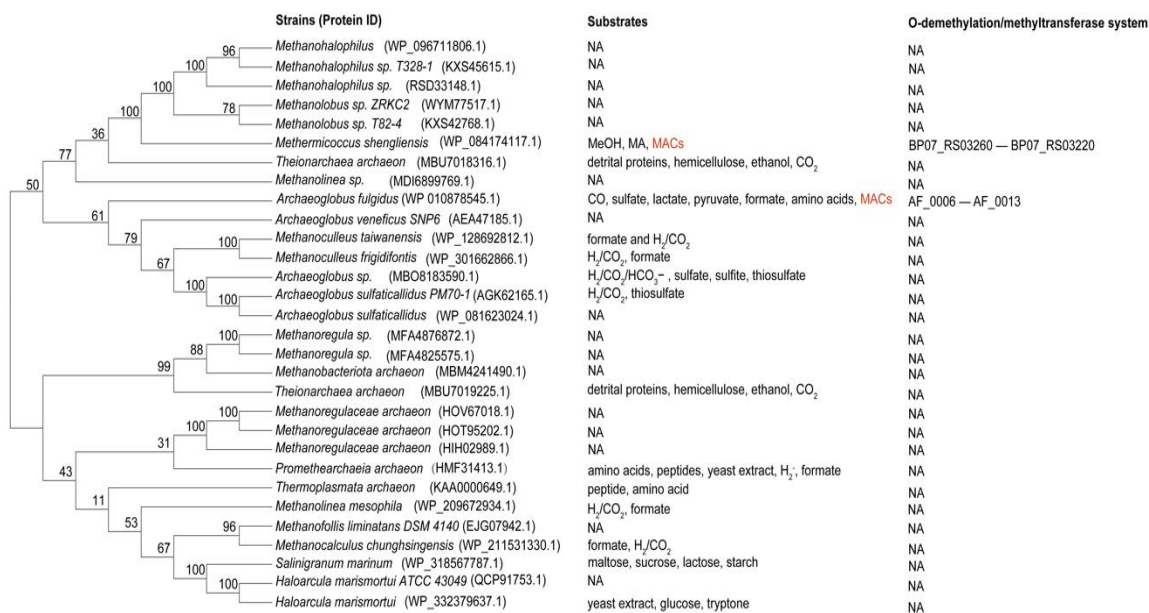


Figure 3-S4. Phylogenetic tree of Tlp. A phylogenetic tree based on the full-length amino acid sequences of selected Tlp proteins was constructed using the Maximum likelihood analysis method with 1000 bootstrap replications in MEGA 11 software. Note that the red pentagram indicates that archaea can use MACs to produce methane metabolism, and species that have been reported in the literature. The gene clusters depicted here encode proteins involved in O-demethoxylation/methyltransferase system (BP07_RS03260-BP07_RS03220; AF_0006-AF_0013). MeOH: methanol; MA: methylamines; MACs: Methoxylated aromatic compounds.

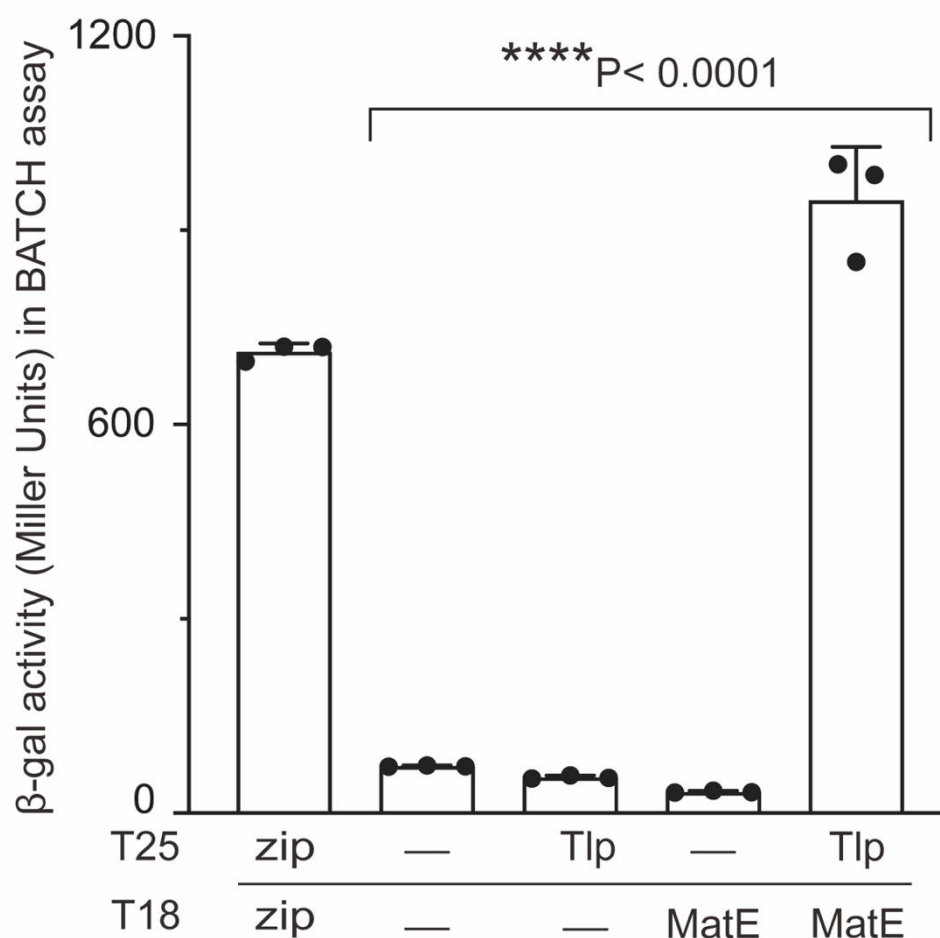


Figure 3-S5. A β-galactosidase assay was performed to assess the interactions between Tlp and MatE. All data represent the mean of n=3 biologically independent samples and error bars show standard deviation, and asterisks indicate statistically significant differences (**** $p < 0.0001$).

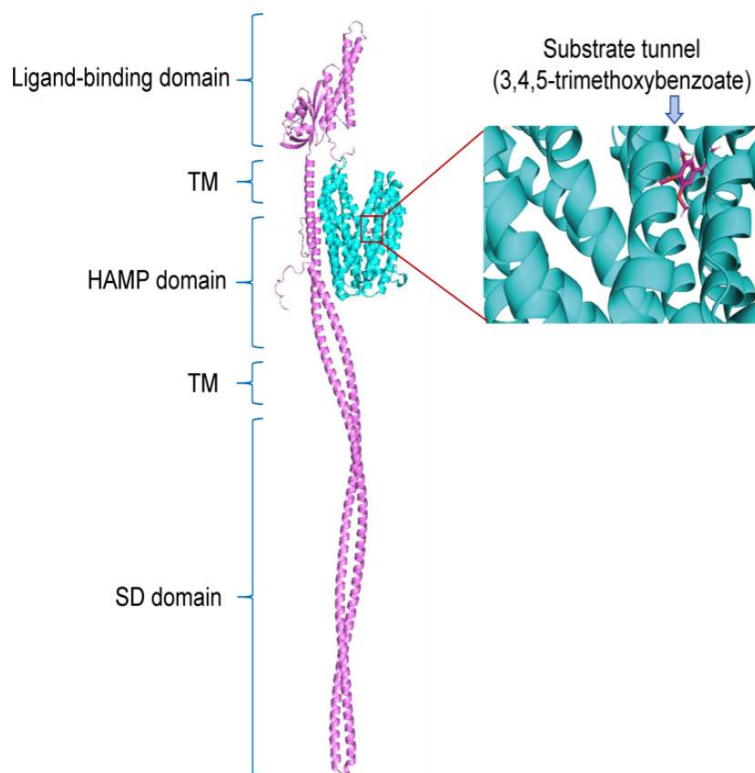


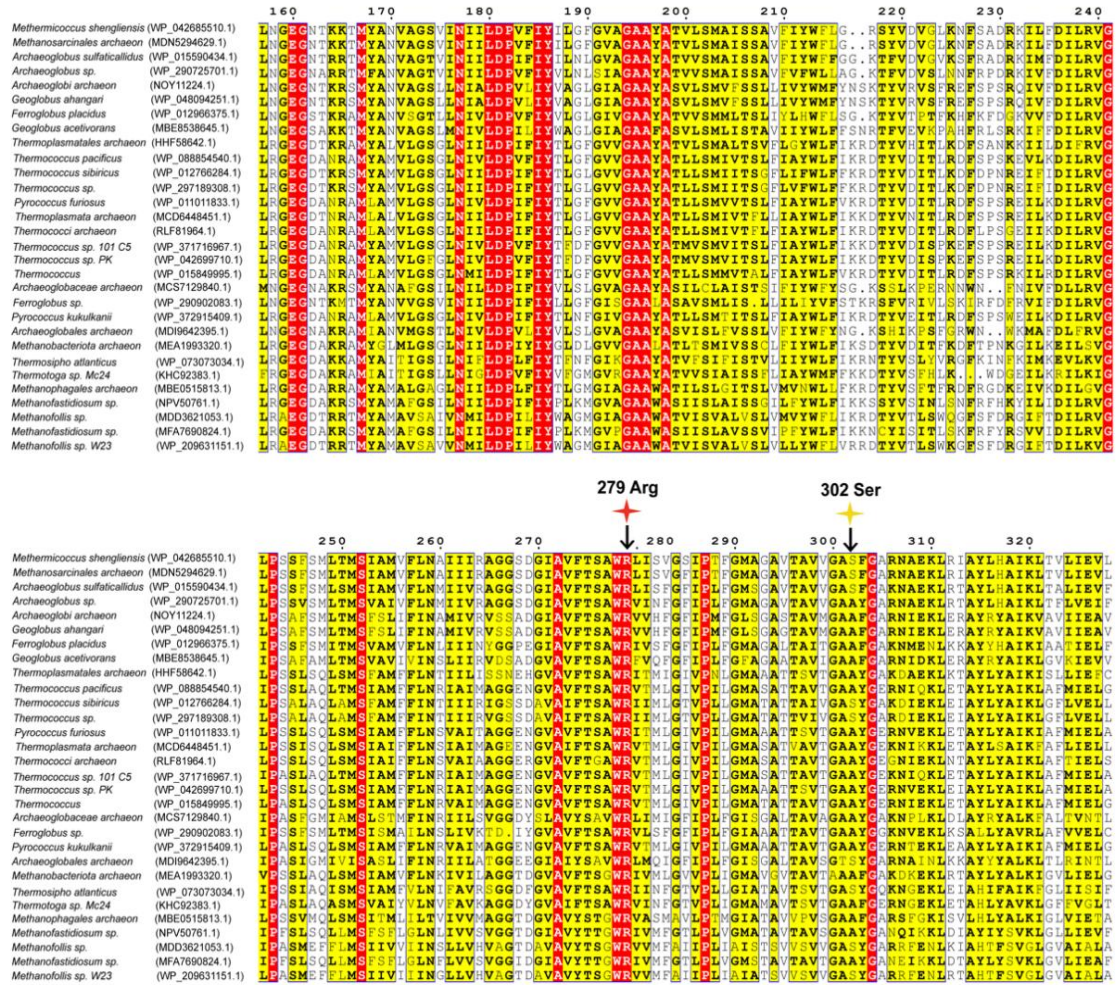
Figure 3-S6. Ligand-MatE interactions in the middle membrane region of the transportation cavity. Molecular docking methods identified 3,4,5-trimethoxybenzoate (purple) located both on the surface and at the center of the protein cavity.

Chapter 3. MatE transporter affects methane metabolism in *Methermicoccus shengliensis* and is modulated by methoxylated aromatic compounds

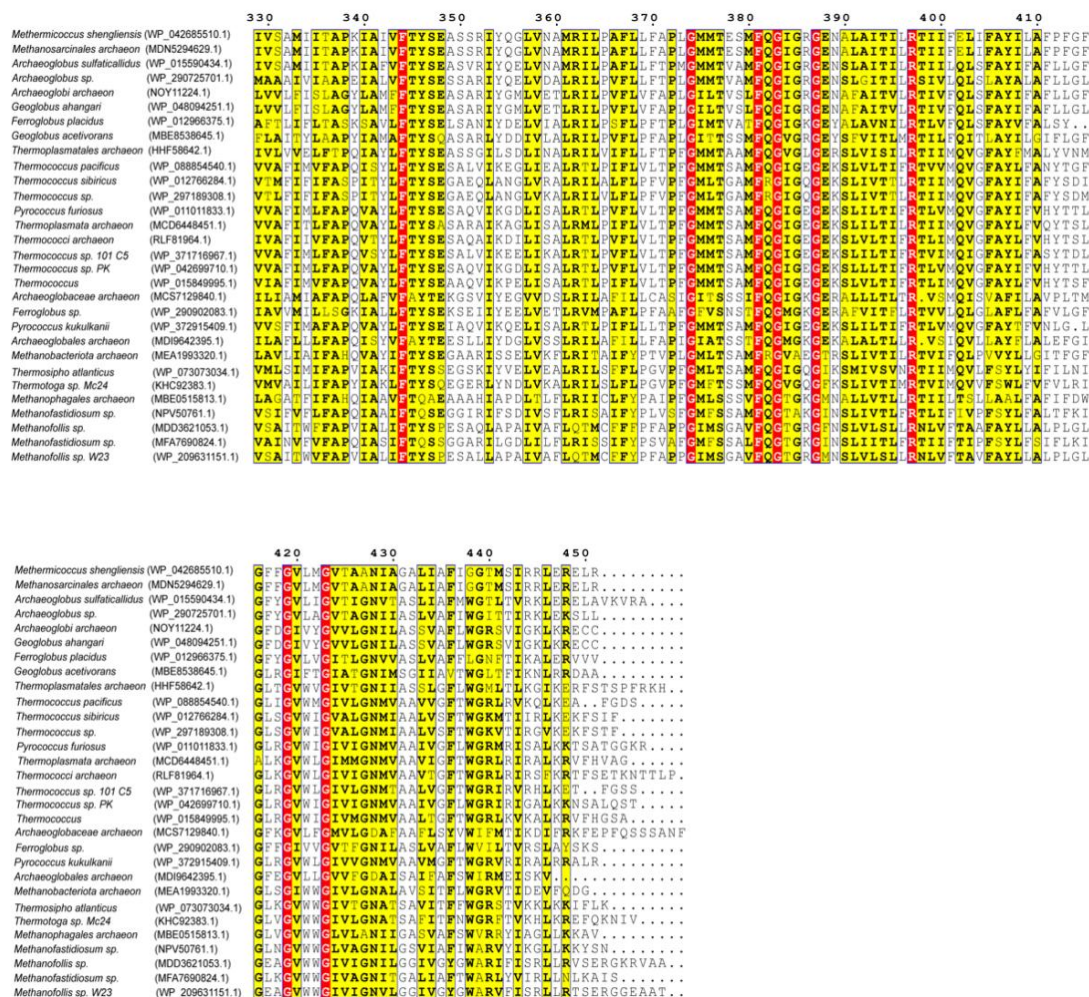
	1	10	20	30	40	50	60	70
<i>Methermicoccus shengliensis</i> (WP_04285510.1)	MKEL	RGV	VLT	GDPKRALVKLSH	MMISNLV	TLYNLADGA	VACLGAD	SAVGIFPLFMVFLSLMG
<i>Methanosarcinales archaeon</i> (MDN5294629.1)	MKEL	RGV	VLT	GDPKRALVKLSH	MMISNLV	TLYNLADGA	VACLGAD	SAVGIFPLFMVFLSLMG
<i>Archaeoglobus sulfatocalidus</i> (WP_015590434.1)	MKEL	RGV	VLT	GDPKRALVKLSH	MMISNLV	TLYNLADGA	VACLGAD	SAVGIFPLFMVFLSLMG
<i>Archaeoglobus</i> sp. (WP_290725701.1)	MKEL	RGV	VLT	GDPKRALVKLSH	MMISNLV	TLYNLADGA	VACLGAD	SAVGIFPLFMVFLSLMG
<i>Archaeoglobi archaeon</i> (NOY11224.1)	MRRRT	RGV	VLT	GDPKRALVKLSH	MMISNLV	TLYNLADGA	VACLGAD	SAVGIFPLFMVFLSLMG
<i>Geoglobus shangari</i> (WP_048094251.1)	MRRRT	RGV	VLT	GDPKRALVKLSH	MMISNLV	TLYNLADGA	VACLGAD	SAVGIFPLFMVFLSLMG
<i>Ferroglobus placidus</i> (WP_012966375.1)	...M	RGV	VLT	GDPKRALVKLSH	MMISNLV	TLYNLADGA	VACLGAD	SAVGIFPLFMVFLSLMG
<i>Geoglobus acetivorans</i> (MBE8538645.1)	MMKT	RGV	VLT	GDPKRALVKLSH	MMISNLV	TLYNLADGA	VACLGAD	SAVGIFPLFMVFLSLMG
<i>Thermoplasmatales archaeon</i> (MHF58642.1)	MSER	ATKGV	LLR	GDPKRALVKLSH	MMISNLV	TLYNLADGA	VACLGAD	SAVGIFPLFMVFLSLMG
<i>Thermococcus pacificus</i> (WP_088854540.1)	MGEK	VTKGV	LLR	GDPKRALVKLSH	MMISNLV	TLYNLADGA	VACLGAD	SAVGIFPLFMVFLSLMG
<i>Thermococcus sibiricus</i> (WP_012766284.1)	MSQK	VTKGV	LLR	GDPKRALVKLSH	MMISNLV	TLYNLADGA	VACLGAD	SAVGIFPLFMVFLSLMG
<i>Thermococcus</i> sp. (WP_297189308.1)	MSQK	VTKGV	LLR	GDPKRALVKLSH	MMISNLV	TLYNLADGA	VACLGAD	SAVGIFPLFMVFLSLMG
<i>Pyrococcus furiosus</i> (WP_011011833.1)	MSEK	TTKGV	LLR	GDPKRALVKLSH	MMISNLV	TLYNLADGA	VACLGAD	SAVGIFPLFMVFLSLMG
<i>Thermoplasmata archaeon</i> (MCD6448451.1)	MNEK	VTKGV	LLR	GDPKRALVKLSH	MMISNLV	TLYNLADGA	VACLGAD	SAVGIFPLFMVFLSLMG
<i>Thermococci archaeon</i> (RLF81964.1)	MSEK	TTKGV	LLR	GDPKRALVKLSH	MMISNLV	TLYNLADGA	VACLGAD	SAVGIFPLFMVFLSLMG
<i>Thermococcus</i> sp. 101 C5 (WP_371716967.1)	MSEK	ATKGV	LLR	GDPKRALVKLSH	MMISNLV	TLYNLADGA	VACLGAD	SAVGIFPLFMVFLSLMG
<i>Thermococcus</i> sp. PK (WP_042699710.1)	MSEK	ATKGV	LLR	GDPKRALVKLSH	MMISNLV	TLYNLADGA	VACLGAD	SAVGIFPLFMVFLSLMG
<i>Thermococcus</i> (MCS7129840.1)	MSEK	MTKGV	LLR	GDPKRALVKLSH	MMISNLV	TLYNLADGA	VACLGAD	SAVGIFPLFMVFLSLMG
<i>Archaeoglobaceae archaeon</i> (MCS7129840.1)	MLES	KEI	TTT	GDPKRALVKLSH	MMISNLV	TLYNLADGA	VACLGAD	SAVGIFPLFMVFLSLMG
<i>Ferroglobus</i> sp. (WP_290902083.1)	...	...	...	PKLSR	IIISNLAF	TYNFA	GVACLGAD	SAVGIFPLFMVFLSLMG
<i>Pyrococcus kulkarnii</i> (WP_372915409.1)	MSGK	MTKGV	LLR	GDPKRALVKLSH	MMISNLV	TLYNLADGA	VACLGAD	SAVGIFPLFMVFLSLMG
<i>Archaeoglobales archaeon</i> (MDI9642395.1)	MNER	EGTII	ASPR	RALLKLSH	MMISNLV	TLYNLADGA	VACLGAD	SAVGIFPLFMVFLSLMG
<i>Methanobacteriota archaeon</i> (MEA1993320.1)	MSDE	VTG	GV	LLR	GDPKRALVKLSH	MMISNLV	TLYNLADGA	SAVGIFPLFMVFLSLMG
<i>Thermosipho atlanticus</i> (WP_073073034.1)	...	...	...	ETG	GV	LLR	GDPKRALVKLSH	MMISNLV
<i>Thermotoga</i> sp. Mc24 (KHC92383.1)	MER	ETG	GV	LLR	GDPKRALVKLSH	MMISNLV	TLYNLADGA	SAVGIFPLFMVFLSLMG
<i>Methanophagales archaeon</i> (MBE0515813.1)	MPASRTE	EVMSA	ELTPK	GETGV	LLR	GDPKRALVKLSH	MMISNLV	TLYNLADGA
<i>Methanofastidiosum</i> sp. (NPV50761.1)	MSNE	IVNDQ	EIK	...	HTG	GI	LLR	GDPKRALVKLSH
<i>Methanofastidiosum</i> sp. (MDD3621053.1)	MHAT	ETKH	PEQT	...	GTT	RGV	LLT	GDPKRALVKLSH
<i>Methanofastidiosum</i> sp. (MFA7690824.1)	MSEN	IVQNQ	QKK	...	QTD	GI	LLR	GDPKRALVKLSH
<i>Methanofastidiosum</i> sp. W23 (WP_209631151.1)	MHAT	ETKH	PEQT	...	GTT	RGV	LLT	GDPKRALVKLSH

	80	90	100	110	120	130	140	150
<i>Methermicoccus shengliensis</i> (WP_04285510.1)	VGIGASSA	ISRRIC	AGDKRGADNI	MHAIV	GFAAALIL	TLTIIR	LEHLLK	LLGAGGKVL
<i>Methanosarcinales archaeon</i> (MDN5294629.1)	VGIGASSA	ISRRIC	AGDKRGADNI	MHAIV	GFAAALIL	TLTIIR	LEHLLK	LLGAGGKVL
<i>Archaeoglobus sulfatocalidus</i> (WP_015590434.1)	LGIGASSA	ISRRIC	AGDKRGADNI	MHAIV	GFAAALIL	TLTIIR	LEHLLK	LLGAGGKVL
<i>Archaeoglobus</i> sp. (WP_290725701.1)	LGIGASSA	ISRRIC	AGDKRGADNI	MHAIV	GFAAALIL	TLTIIR	LEHLLK	LLGAGGKVL
<i>Archaeoglobi archaeon</i> (NOY11224.1)	LGIGTSSA	ISRRIC	AGDKRGADNI	MHAIV	GFAAALIL	TLTIIR	LEHLLK	LLGAGGKVL
<i>Geoglobus shangari</i> (WP_048094251.1)	LGIGTSSA	ISRRIC	AGDKRGADNI	MHAIV	GFAAALIL	TLTIIR	LEHLLK	LLGAGGKVL
<i>Ferroglobus placidus</i> (WP_012966375.1)	LGIGASSA	ISRRIC	AGDKRGADNI	MHAIV	GFAAALIL	TLTIIR	LEHLLK	LLGAGGKVL
<i>Geoglobus acetivorans</i> (MBE8538645.1)	LGIGASSA	ISRRIC	AGDKRGADNI	MHAIV	GFAAALIL	TLTIIR	LEHLLK	LLGAGGKVL
<i>Thermoplasmatales archaeon</i> (MHF58642.1)	LGIGASSA	ISRRIC	AGDKRGADNI	MHAIV	GFAAALIL	TLTIIR	LEHLLK	LLGAGGKVL
<i>Thermococcus pacificus</i> (WP_088854540.1)	LGIGASSA	ISRRIC	AGDKRGADNI	MHAIV	GFAAALIL	TLTIIR	LEHLLK	LLGAGGKVL
<i>Thermococcus sibiricus</i> (WP_012766284.1)	LGIGASSA	ISRRIC	AGDKRGADNI	MHAIV	GFAAALIL	TLTIIR	LEHLLK	LLGAGGKVL
<i>Thermococcus</i> sp. (WP_297189308.1)	LGIGTSSA	ISRRIC	AGDKRGADNI	MHAIV	GFAAALIL	TLTIIR	LEHLLK	LLGAGGKVL
<i>Pyrococcus furiosus</i> (WP_011011833.1)	LGIGASSA	ISRRIC	AGDKRGADNI	MHAIV	GFAAALIL	TLTIIR	LEHLLK	LLGAGGKVL
<i>Thermoplasmata archaeon</i> (MCD6448451.1)	LGIGASSA	ISRRIC	AGDKRGADNI	MHAIV	GFAAALIL	TLTIIR	LEHLLK	LLGAGGKVL
<i>Thermococci archaeon</i> (RLF81964.1)	LGIGASSA	ISRRIC	AGDKRGADNI	MHAIV	GFAAALIL	TLTIIR	LEHLLK	LLGAGGKVL
<i>Thermococcus</i> sp. 101 C5 (WP_371716967.1)	LGIGASSA	ISRRIC	AGDKRGADNI	MHAIV	GFAAALIL	TLTIIR	LEHLLK	LLGAGGKVL
<i>Thermococcus</i> sp. PK (WP_042699710.1)	LGIGASSA	ISRRIC	AGDKRGADNI	MHAIV	GFAAALIL	TLTIIR	LEHLLK	LLGAGGKVL
<i>Thermococcus</i> (MCS7129840.1)	LGIGASSA	ISRRIC	AGDKRGADNI	MHAIV	GFAAALIL	TLTIIR	LEHLLK	LLGAGGKVL
<i>Archaeoglobaceae archaeon</i> (MCS7129840.1)	LGIGASSA	ISRRIC	AGDKRGADNI	MHAIV	GFAAALIL	TLTIIR	LEHLLK	LLGAGGKVL
<i>Ferroglobus</i> sp. (WP_290902083.1)	LGIGASSA	ISRRIC	AGDKRGADNI	MHAIV	GFAAALIL	TLTIIR	LEHLLK	LLGAGGKVL
<i>Pyrococcus kulkarnii</i> (WP_372915409.1)	LGIGASSA	ISRRIC	AGDKRGADNI	MHAIV	GFAAALIL	TLTIIR	LEHLLK	LLGAGGKVL
<i>Archaeoglobales archaeon</i> (MDI9642395.1)	LSVASSA	ISRRIC	AGDKRGADNI	MHAIV	GFAAALIL	TLTIIR	LEHLLK	LLGAGGKVL
<i>Methanobacteriota archaeon</i> (MEA1993320.1)	LGIGASSA	ISRRIC	AGDKRGADNI	MHAIV	GFAAALIL	TLTIIR	LEHLLK	LLGAGGKVL
<i>Thermosipho atlanticus</i> (WP_073073034.1)	LGIGASSA	ISRRIC	AGDKRGADNI	MHAIV	GFAAALIL	TLTIIR	LEHLLK	LLGAGGKVL
<i>Thermotoga</i> sp. Mc24 (KHC92383.1)	LGIGASSA	ISRRIC	AGDKRGADNI	MHAIV	GFAAALIL	TLTIIR	LEHLLK	LLGAGGKVL
<i>Methanophagales archaeon</i> (MBE0515813.1)	LGIGASSA	ISRRIC	AGDKRGADNI	MHAIV	GFAAALIL	TLTIIR	LEHLLK	LLGAGGKVL
<i>Methanofastidiosum</i> sp. (NPV50761.1)	LGIGASSA	ISRRIC	AGDKRGADNI	MHAIV	GFAAALIL	TLTIIR	LEHLLK	LLGAGGKVL
<i>Methanofastidiosum</i> sp. (MDD3621053.1)	LGIGASSA	ISRRIC	AGDKRGADNI	MHAIV	GFAAALIL	TLTIIR	LEHLLK	LLGAGGKVL
<i>Methanofastidiosum</i> sp. (MFA7690824.1)	LGIGASSA	ISRRIC	AGDKRGADNI	MHAIV	GFAAALIL	TLTIIR	LEHLLK	LLGAGGKVL
<i>Methanofastidiosum</i> sp. W23 (WP_209631151.1)	LGIGASSA	ISRRIC	AGDKRGADNI	MHAIV	GFAAALIL	TLTIIR	LEHLLK	LLGAGGKVL

Functional characterization of a MatE transporter in *Methermicoccus shengliensis* and evolutionary diversity of archaeal MatE transporters



Chapter 3. MatE transporter affects methane metabolism in *Methermicoccus shengliensis* and is modulated by methoxylated aromatic compounds



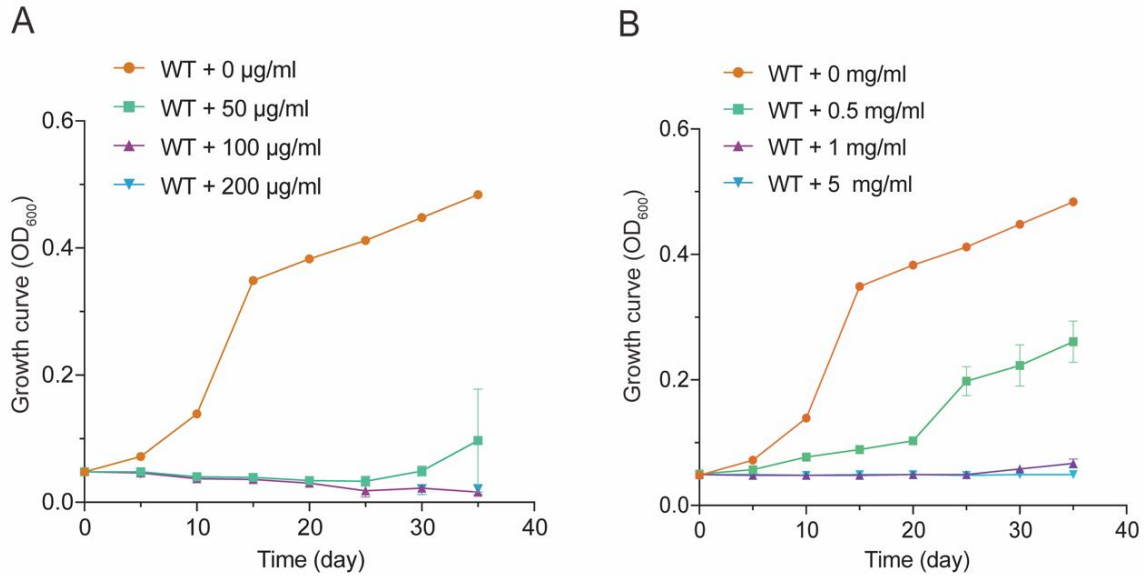


Figure 3-S8. Minimal inhibitory concentration determination. **A.** Growth curves of *M. shengliensis* under methanol containing varied concentrations of nourseothricin (0 - 200 µg·mL⁻¹). **B.** Growth curves of *M. shengliensis* under methanol containing varied concentrations of neomycin (0 - 5 mg·mL⁻¹). All data represent the mean of n=3 biologically independent samples (**A**, **B**).

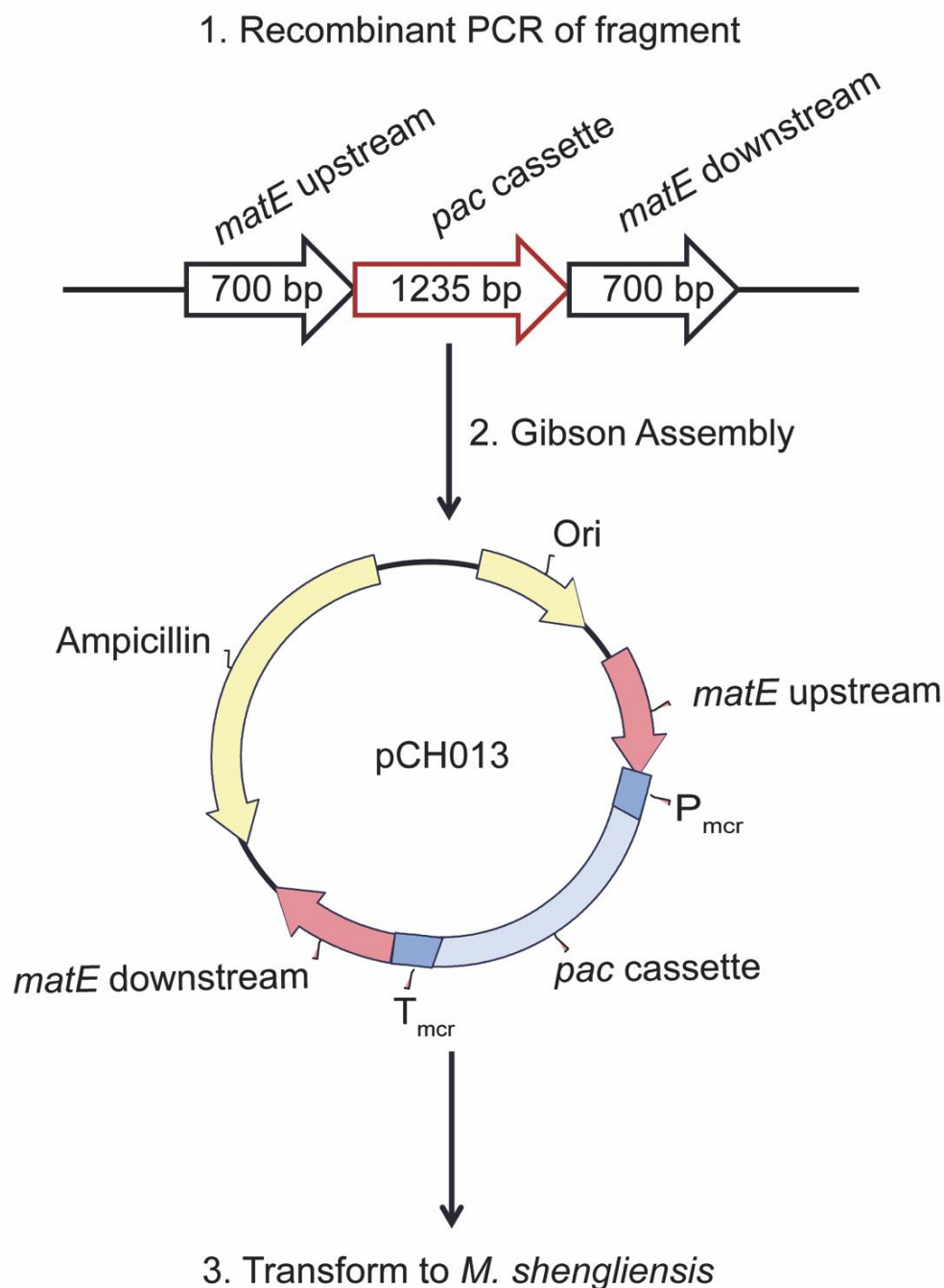


Figure 3-S9. Map of the integration plasmid pCH013 used for gene replacement. The vector contains the puromycin resistance marker (Puro) for positive selection, an ampicillin resistance cassette (Amp) for *E. coli*, an origin of replication (Ori) for *E. coli*, the methyl coenzyme M reductase promoter (P_{mcr}) from *Methanococcus voltae*, and the methyl coenzyme M reductase terminator (T_{mcr}) from *M. voltae*.

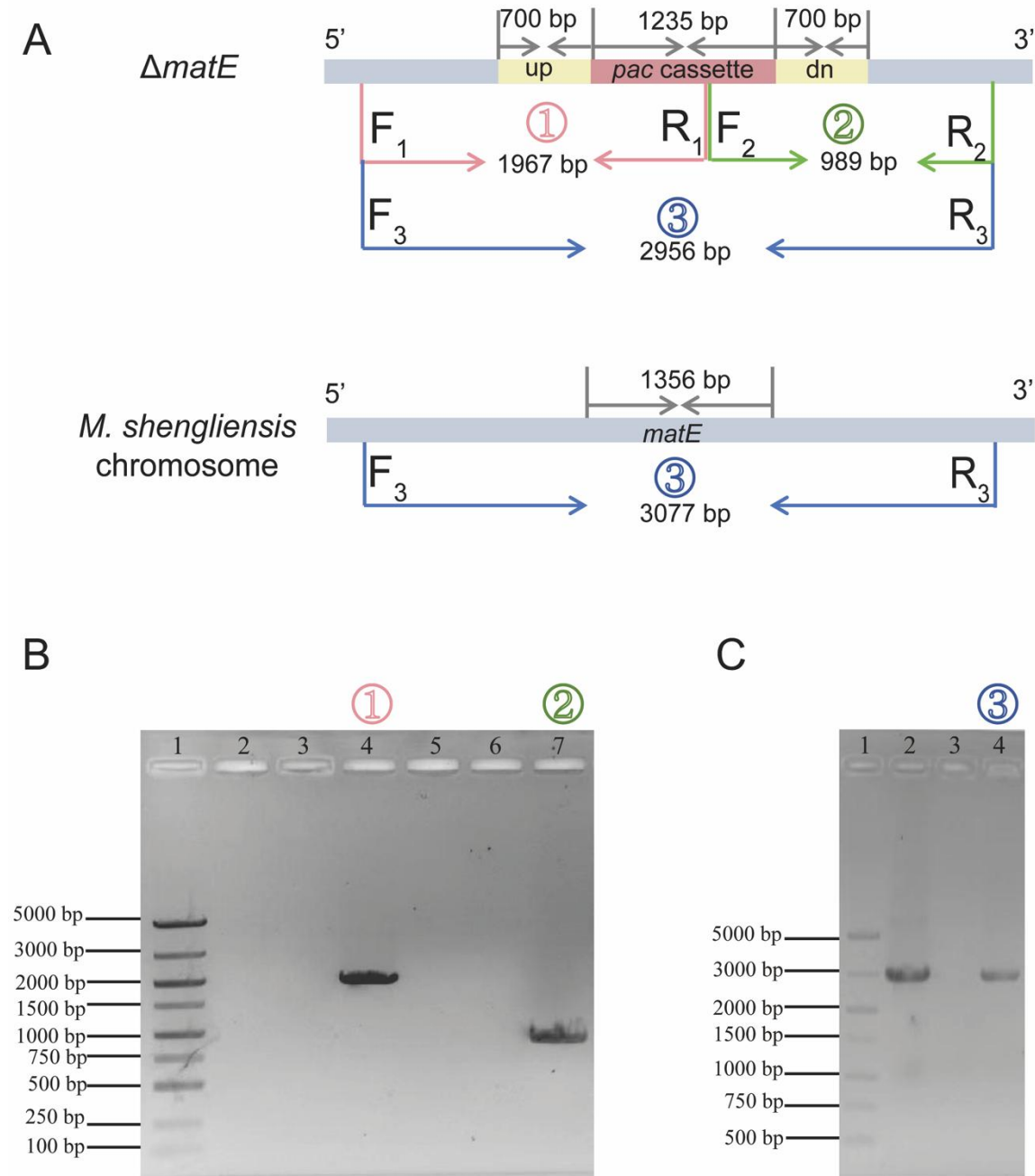
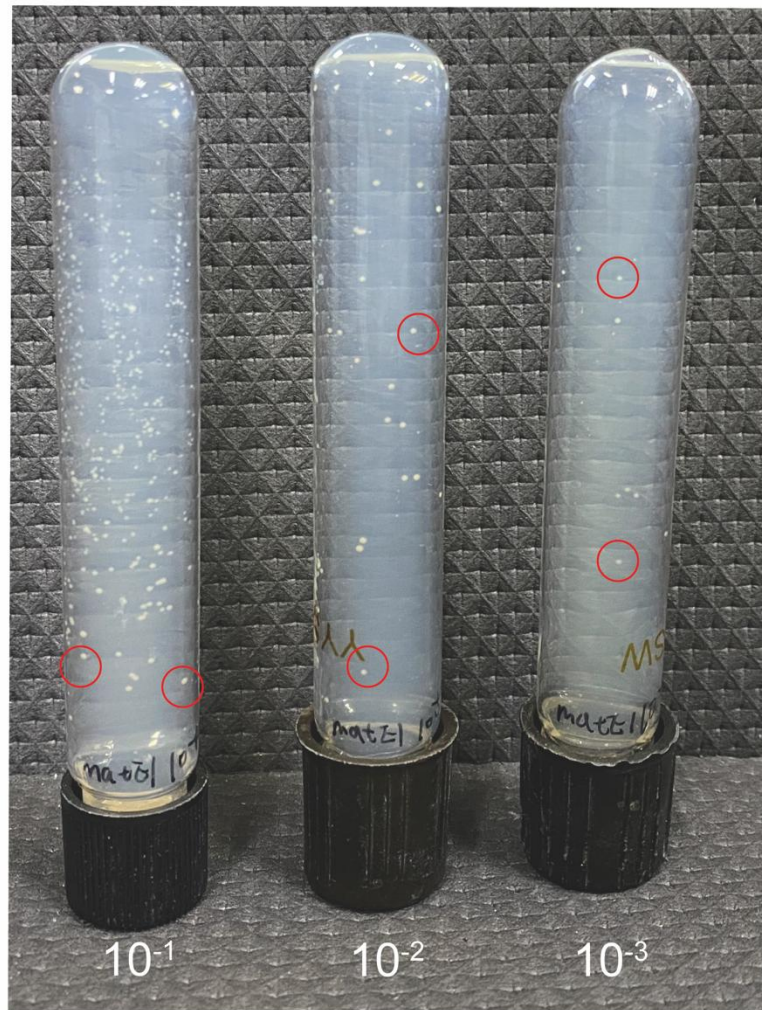


Figure 3-S10. PCR validation of the $\Delta matE$ mutant. **A.** A schematic of PCR validation for the $\Delta matE$ mutant. Note that the blue rectangular box represents the genomic sequence of *M. shengliensis*, the yellow rectangular box represents the upstream and downstream sequence of the recombinant plasmid, and the red rectangular box represents the antibiotic resistance gene sequence. ①, ②, ③ represent three different pairs of primers. **B.** PCR verification of *M. shengliensis* $\Delta matE$ mutant. Primer ①: F₁, R₁ (1967 bp) and primer ②: F₂, R₂ (989 bp) were selected one primer binds within *pac*, and

the other binds to the chromosome outside of the homologous recombination regions for PCR verification. Gel image: Lane 1, Marker (5000 bp); Lane 2 and 5, Control 1 (*M. shengliensis* chromosome); Lane 3 and 6, Control 2 (recombinant plasmid); Lane 4 and 7, $\Delta matE$ mutant (1967 bp and 989 bp). C. PCR verification of *M. shengliensis* $\Delta matE$ mutant. Primer ③: F₃, R₃ binding to the chromosome of *M. shengliensis* outside of the homologous recombination regions to validate the $\Delta matE$ mutant by PCR. Gel image: Lane 1, Marker (5000 bp); Lane 2, Control 1 (*M. shengliensis* chromosome: 3077 bp); Lane 3, Control 2 (recombinant plasmid); Lane 4, $\Delta matE$ mutant (2956 bp). The sequence alignment results of the PCR products after sequencing are presented on pages 19 to 29 at the end of this document.

A



B

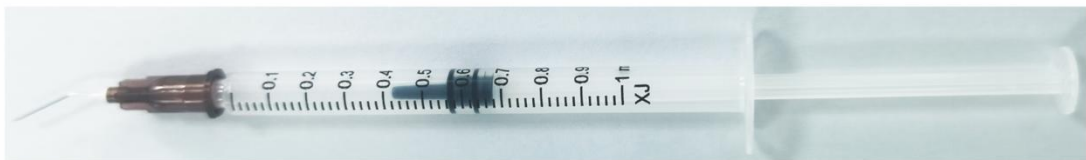


Figure 3-S11. Scheme of picking colonies from agar Hungate tubes at the anaerobic glove box. **A.** A 10 mL Hungate tubes plated with *M. shengliensis* forms colonies after 10 days. After opening, a sterile N₂ gassing cannula was introduced to prevent air from entering the tube and to maintain anaerobiosis. **B.** To pick the colony more accurately, the needle was bent about 45 °C by folding the end of the needle against the needle cap. A 1 mL syringe with a 1-inch needle was used for transferring the colony. Enlarged image of the 1 mL syringe with the bent needle.

Chapter 3. MatE transporter affects methane metabolism in *Methermicoccus shengliensis* and is modulated by methoxylated aromatic compounds

```

RP -----
MT GACAACGTGAAGCCGAAGGGAATATGCGGGAGCGGGATGATCGACCTCGTGGCTGGGCTC

RP -----
MT TACAAAGCCGGGATAATAAACGGAAGGGGAAGTTCGTCAGAGAGACTGAGAGGATAATT

RP -----AACCGAGACGGGAAAGGAG
MT AAAGACCGCGTTCCAAAATTCGTTGTTGCGTGGGCAGATGAAACCGAGACGGGAAAGGAG
      *****

RP ATTACAGTTACCGAAAAGGACATAAACGAGCTTCTGATGGCAAAGGGGGCTATAAGGTCT
MT ATTACAGTTACCGAAAAGGACATAAACGAGCTTCTGATGGCAAAGGGGGCTATAAGGTCT
      *****

RP GGATGGATGATTCTGATGGAGAAGCTCGGAATTGAAGCGGAGAATATTTCTAGGATATAT
MT GGATGGATGATTCTGATGGAGAAGCTCGGAATTGAAGCGGAGAATATTTCTAGGATATAT
      *****

RP TTGGCTGGCTCCTTTGGAAGGCACATAAAATATCGAGAACGCGAAGCTCATCGGACTTCTG
MT TTGGCTGGCTCCTTTGGAAGGCACATAAAATATCGAGAACGCGAAGCTCATCGGACTTCTG
      *****

RP CCAGATGTTCCAAATGACAGAATTGTCTTTGCTGGAGACACAGCTGTTGGTGGTGCAAAG
MT CCAGATGTTCCAAATGACAGAATTGTCTTTGCTGGAGACACAGCTGTTGGTGGTGCAAAG
      *****

RP ATGGCTCTGAAATCTGTTAAGGAGAGAGAGGAGATTGAGGAGGTTGTTGACTTTGTCGAG
MT ATGGCTCTGAAATCTGTTAAGGAGAGAGAGGAGATTGAGGAGGTTGTTGACTTTGTCGAG
      *****

RP TATGTCGAGCTTTCGTTGACAGGAACTTTACCGAACTTTCGTAAGGGCGATTCCGATA
MT TATGTCGAGCTTTCGTTGACAGGAACTTTACCGAACTTTCGTAAGGGCGATTCCGATA
      *****

RP TCGCAAATTTTAAATTCCAAAAAGTTTCAGGTGAAAAATGGAGAAAACTCTCTTCATGA
MT TCGCAAATTTTAAATTCCAAAAAGTTTCAGGTGAAAAATGGAGAAAACTCTCTTCATGA
      *****

RP TCCTTTAAACATGAAAAAGCACATAATTAGGGAGCTTCCGGAGAGCTGCCCCTCCTTG
MT TCCTTTAAACATGAAAAAGCACATAATTAGGGAGCTTCCGGAGAGCTGCCCCTCCTTG
      *****

RP AGTTTAAATGTCTCAAAAAGAGATAATCGAGGAGACAAGATTGACGAAAGGGACGGTATC
MT AGTTTAAATGTCTCAAAAAGAGATAATCGAGGAGACAAGATTGACGAAAGGGACGGTATC
      *****

RP AAAGAAAGAAAGCGGTGATGAAAAAATTACCTCAGAGATTAACGAGATTCTCTTGAAAAA
MT AAAGAAAGAAAGCGGTGATGAAAAAATTACCTCAGAGATTAACGAGATTCTCTTGAAAAA
      *****

RP CTTACCGAAGATGAAAAGCGGACTCTGCAAACCTTCTGAAAAAATGCTGAGAAATAT
MT CTTACCGAAGATGAAAAGCGGACTCTGCAAACCTTCTGAAAAAATGCTGAGAAATAT
      *****

RP TGAGCTTACGGGTGATGTGGCCGCTGCAGATAAAAAACGCCCTATTCGTAATATACTATT
MT TGAGCTTACGGGTGATGTGGCCGCTGCAGATAAAAAACGCCCTATTCGTAATATACTATT
      *****

RP CAAAACTAAATATTCATTGAAATATTTGGGTAATAATTAATATTAATCTAATATTTAGAT
MT CAAAACTAAATATTCATTGAAATATTTGGGTAATAATTAATATTAATCTAATATTTAGAT
      *****

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Functional characterization of a MatE transporter in *Methermicoccus shengliensis* and evolutionary diversity of archaeal MatE transporters

RP	ATTGAGATATAATAATAACATTTTTTTTAAAAAATCATCTATTTTCATCTTAATTTTATAT
MT	ATTGAGATATAATAATAACATTTTTTTTAAAAAATCATCTATTTTCATCTTAATTTTATAT *****
RP	ATTAAAGCCCATTTTTTGAGTATTCAAATTCAAATTATTGTGTTATTAACATCTTATATA
MT	ATTAAAGCCCATTTTTTGAGTATTCAAATTCAAATTATTGTGTTATTAACATCTTATATA *****
RP	TAAACTTTTCTATTTAATGTTAATGAAAAAGTGAATATATATACATAGAGTAATGTTATG
MT	TAAACTTTTCTATTTAATGTTAATGAAAAAGTGAATATATATACATAGAGTAATGTTATG *****
RP	ATGTATATATCAAAAAAACTAGAGGAGACGACCTTCCATGACAGAATATAAACCAACAG
MT	ATGTATATATCAAAAAAACTAGAGGAGACGACCTTCCATGACAGAATATAAACCAACAG *****
RP	TTAGATTAGCAACAAGAGATGACGTACCTAGGGCAGTAAGAACTCTTGCAGCAGCTTTTG
MT	TTAGATTAGCAACAAGAGATGACGTACCTAGGGCAGTAAGAACTCTTGCAGCAGCTTTTG *****
RP	CAGATTATCCAGCAACAAGACACACAGTTGATCCAGATAGACACATTGAAAGAGTTACAG
MT	CAGATTATCCAGCAACAAGACACACAGTTGATCCAGATAGACACATTGAAAGAGTTACAG *****
RP	AATTACAAGAATTATTTTTTAACAAGAGTTGGTCTTGATATAGGTAAAGTATGGGTGCAG
MT	AATTACAAGAATTATTTTTTAACAAGAGTTGGTCTTGATATAGGTAAAGTATGGGTGCAG *****
RP	ATGATGGAGCAGCAGTTGCAGTTTGGACAACACCAGAATCAGTTGAAGCTGGTGCTGTTT
MT	ATGATGGAGCAGCAGTTGCAGTTTGGACAACACCAGAATCAGTTGAAGCTGGTGCTGTTT *****
RP	TTGCAGAAATTGGACCAAGAATGGCTGAATTATCAGGATCAAGATTAGCAGCTCAGCAAC
MT	TTGCAGAAATTGGACCAAGAATGGCTGAATTATCAGGATCAAGATTAGCAGCTCAGCAAC *****
RP	AGATGGAAGGATTACTTGCAACACACAGACCAAAAGAACCAGCATGGTTTTTAGCAACAG
MT	AGATGGAAGGATTACTTGCAACACACAGACCAAAAGAACCAGCATGGTTTTTAGCAACAG *****
RP	TTGGAGTTTCACCAGATCACCAGGGAAGGTTTAGGTTCTGCTGTTGTACTTCCTGGTG
MT	TTGGAGTTTCACCAGATCACCAGGGAAGGTTTAGGTTCTGCTGTTGTACTTCCTGGTG *****
RP	TTGAAGCAGCTGAAAGAGCAGGAGTTCCAGCATTTTTTAGAAACATCAGCACCAAGAAATC
MT	TTGAAGCAGCTGAAAGAGCAGGAGTTCCAGCATTTTTTAGAAACATCAGCACCAAGAAATC *****
RP	TTCCATTTTATGAAAGACTTGGATTTACAGTTACAGCAGATGTTGAAGTTCCAGAAGGAC
MT	TTCCATTTTATGAAAGACTTGGATTTACAGTTACAGCAGATGTTGAAGTTCCAGAAGGAC *****
RP	CAAGAACATGGTGTATGACAAGAAAACCAGGAGCATAACCATGGCTCCGACCGAAGCCAC
MT	CAAGAACATGGTGTATGACAAGAAAACCAGGAGCATAACCATGGCTCCGACCGAAGCCAC *****
RP	CCGGGGCGGCCCGCCGACCCCGACCCGCCCCGAGGCCACCGCGGGGGACACACCGA
MT	CCGGGGCGGCCCGCCGACCCCGACCCGCCCCGAGGCCACCGCGGGGGACACACCGA *****
RP	ACACGCCGACCCTGCTGAACACGCGGCGCAGTTCGGTGCCAGGAGCGGATCGGGAATTA
MT	ACACGCCGACCCTGCTGAACACGCGGCGCAGTTCGGTGCCAGGAGCGGATCGGGAATTA

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*****
RP      ATTCGAAGCTGCTGGTGAAAGAGACCCTATCTTACCTGCTAAAATCT
MT      ATTCGAAGCTGCTGGTGAAAGAGACCCTATCTTACCTGCTAAAATCT
*****
```

Figure 3-S12. The sequencing alignment results of the $\Delta matE$ mutants. Note that we performed multiple sequence alignment using the CLUSTAL X (1.83) software. Showed that the mutant successfully exhibited the wild-type chromosomal sequence outside the upstream region of the homologous recombinant plasmid.

Functional characterization of a MatE transporter in *Methermicoccus shengliensis* and evolutionary diversity of archaeal MatE transporters

RP	GAGACCCTATCTTACCTGCTAAAATCTAAGTTAATTACTAATTTATTATTAATTTATTAT
MT	GAGACCCTATCTTACCTGCTAAAATCTAAGTTAATTACTAATTTATTATTAATTTATTAT *****
RP	TAGATTGGGTAAAATAGTAAAAGAAAACCTAAAGGAAACCTAATATGGTTTCCTTTTTTTTA
MT	TAGATTGGGTAAAATAGTAAAAGAAAACCTAAAGGAAACCTAATATGGTTTCCTTTTTTTTA *****
RP	TATATTTTAAATTCACCTGGGGGGATTTAAAACCTCAAGGTGAGAGTATGAGGAGAGCGGC
MT	TATATTTTAAATTCACCTGGGGGGATTTAAAACCTCAAGGTGAGAGTATGAGGAGAGCGGC *****
RP	GCTTTTAAACAATAACTCTCGCTTCATTCCTAACTCCCTTCGCGCTTTCATCTGTAAATGT
MT	GCTTTTAAACAATAACTCTCGCTTCATTCCTAACTCCCTTCGCGCTTTCATCTGTAAATGT *****
RP	CGCTCTCCCGATGATTGGAAAAGGGTTTGCGGTGGATGCGATAACGCTCAACTGGATTGC
MT	CGCTCTCCCGATGATTGGAAAAGGGTTTGCGGTGGATGCGATAACGCTCAACTGGATTGC *****
RP	TACCGCTTATCTTCTTTCTTCCGCCATGTTTCTCATTCATTCCGTCAGGATAGCTGATAT
MT	TACCGCTTATCTTCTTTCTTCCGCCATGTTTCTCATTCATTCCGTCAGGATAGCTGATAT *****
RP	TAAAGGAAGGAGAATGGTTTTTGTCTCGGAGTGGGTGTTTTACTGCAGGATCAGTGT
MT	TAAAGGAAGGAGAATGGTTTTTGTCTCGGAGTGGGTGTTTTACTGCAGGATCAGTGT *****
RP	CTCAGGCCTATCCACTTCTGCAGAAATGCTGATAGCATTTCAGAGTCCTGCAGGGAATTGG
MT	CTCAGGCCTATCCACTTCTGCAGAAATGCTGATAGCATTTCAGAGTCCTGCAGGGAATTGG *****
RP	AGGTGCAATGGTATTTGCGACAGGAATGGCAATTCTAATCTCCGTTTTCCCGCTAAATGA
MT	AGGTGCAATGGTATTTGCGACAGGAATGGCAATTCTAATCTCCGTTTTCCCGCTAAATGA *****
RP	GAGGGGAAAAGTTTTAGGAATCAATGTGGCTTCAGTTTACACCGGCTTAAGCCTTGGCCC
MT	GAGGGGAAAAGTTTTAGGAATCAATGTGGCTTCAGTTTACACCGGCTTAAGCCTTGGCCC *****
RP	CTTCTTCGCGGCATTTTAACCCAGAACTTCGGATGGAGGAGTGTTTTTCTTGTAATGT
MT	CTTCTTCGCGGCATTTTAACCCAGAACTTCGGATGGAGGAGTGTTTTTCTTGTAATGT *****
RP	CCCTCTCGGCATCATTGTTGTTGCAGTCGCCCTATGGAAGCTGAAAGCGGAGTGGGCTGA
MT	CCCTCTCGGCATCATTGTTGTTGCAGTCGCCCTATGGAAGCTGAAAGCGGAGTGGGCTGA *****
RP	TGCTAAAGAGGAGAGGTTTCGATTGTTGGTCTGCAATTTACAGCCTGATGCTCGTGCT
MT	TGCTAAAGAGGAGAGGTTTCGATTGTTGGTCTGCAATTTACAGCCTGATGCTCGTGCT *****
RP	GATAATGATTGGTGTGTCTGAATCAACCCTGATTCTGATAACAGCGGGCTTGGCTCTGCT
MT	GATAATGATTGGTGTGTCTGAATCAACCCTGATTCTGATAACAGCGGGCTTGGCTCTGCT *****
RP	GATTGTCTTCATTGTC-----
MT	GATTGTCTTCATTGTCGGGAAAGCAGAATCGAACATCCCGTCCTTGAAATAAGGCTTTT *****
RP	-----
MT	CAGGCGAAACATAACTTTTGCATTCTCAAACCTTGCGGCTCTGCTGAACTACGCTGCCAC

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RP      -----
MT      TTTGCGCCGTAACTACCTCTTAAGCCTATACTTGCAGTACATCAAAGCTTTAACGC

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Figure 3-S13. The sequencing alignment results of the $\Delta matE$ mutants. Note that we performed multiple sequence alignment using the CLUSTAL X (1.83) software. RP: recombinant plasmid, MT: mutant strain. Showed that the mutant successfully exhibited the wild-type chromosomal sequence outside the downstream region of the homologous recombinant plasmid.

Functional characterization of a MatE transporter in *Methermicoccus shengliensis* and evolutionary diversity of archaeal MatE transporters

RP	-----
MT	GACAACGTGAAGCCGAAGGGAATATGCGGGAGCGGGATGATCGACCTCGTGGCTGGGCTC
RP	-----
MT	TACAAAGCCGGGATAATAAACGGAAGGGGAAGTTCGTGAGAGAGACTGAGAGGATAATT
RP	-----AACCGAGACGGGAAAGGAG
MT	AAAGACCGCGTTCCAAAATTCGTTGTTGCGTGGGCAGATGAAACCGAGACGGGAAAGGAG *****
RP	ATTACAGTTACCGAAAAGGACATAAACGAGCTTCTGATGGCAAAGGGGGCTATAAGGTCT
MT	ATTACAGTTACCGAAAAGGACATAAACGAGCTTCTGATGGCAAAGGGGGCTATAAGGTCT *****
RP	GGATGGATGATTCTGATGGAGAAGCTCGGAATTGAAGCGGAGAATATTTCTAGGATATAT
MT	GGATGGATGATTCTGATGGAGAAGCTCGGAATTGAAGCGGAGAATATTTCTAGGATATAT *****
RP	TTGGCTGGCTCCTTTGGAAGGCACATAAAATATCGAGAACGCGAAGCTCATCGGACTTCTG
MT	TTGGCTGGCTCCTTTGGAAGGCACATAAAATATCGAGAACGCGAAGCTCATCGGACTTCTG *****
RP	CCAGATGTTCCAAATGACAGAATTGTCTTTGCTGGAGACACAGCTGTTGGTGGTGCAAAG
MT	CCAGATGTTCCAAATGACAGAATTGTCTTTGCTGGAGACACAGCTGTTGGTGGTGCAAAG *****
RP	ATGGCTCTGAAATCTGTTAAGGAGAGAGAGGAGATTGAGGAGGTTGTTGACTTTGTCGAG
MT	ATGGCTCTGAAATCTGTTAAGGAGAGAGAGGAGATTGAGGAGGTTGTTGACTTTGTCGAG *****
RP	TATGTCGAGCTTTCGGTTGACAGGAACTTTACCGAACTTTCGTAAGGGCGATTCCGATA
MT	TATGTCGAGCTTTCGGTTGACAGGAACTTTACCGAACTTTCGTAAGGGCGATTCCGATA *****
RP	TCGCAAATTTTAAATTCCAAAAAGTTTCAGGTGAAAAATGGAGAAAACTCTCTTCATGA
MT	TCGCAAATTTTAAATTCCAAAAAGTTTCAGGTGAAAAATGGAGAAAACTCTCTTCATGA *****
RP	TCCTTTAAACATGAAAAAGCACATAATTAGGGAGCTTCCGGAGAGCTGCCCCCTCCTTG
MT	TCCTTTAAACATGAAAAAGCACATAATTAGGGAGCTTCCGGAGAGCTGCCCCCTCCTTG *****
RP	AGTTTAAATGTCTCAAAAAGAGATAATCGAGGAGACAAGATTGACGAAAGGGACGGTATC
MT	AGTTTAAATGTCTCAAAAAGAGATAATCGAGGAGACAAGATTGACGAAAGGGACGGTATC *****
RP	AAAGAAAGAAAGCGGTGATGAAAAAATTACCTCAGAGATTAACGAGATTCTCTTGAAAAA
MT	AAAGAAAGAAAGCGGTGATGAAAAAATTACCTCAGAGATTAACGAGATTCTCTTGAAAAA *****
RP	CTTACCGAAGATGAAAAGCGGACTCTGCAAACCCTTCTGAAAAAATGCTGAGAAATAT
MT	CTTACCGAAGATGAAAAGCGGACTCTGCAAACCCTTCTGAAAAAATGCTGAGAAATAT *****
RP	TGAGCTTACGGGTGATGTGGCCGCTGCAGATAAAAAACGCCCTATTCTGTAATATACTATT
MT	TGAGCTTACGGGTGATGTGGCCGCTGCAGATAAAAAACGCCCTATTCTGTAATATACTATT *****
RP	CAAACTAAATATTCATTGAAATATTTGGGTAATAATTAATATTAATCTAATATTTAGAT
MT	CAAACTAAATATTCATTGAAATATTTGGGTAATAATTAATATTAATCTAATATTTAGAT *****

RP	ATTGAGATATAATAATAACATTTTTTTAAAAAATCATCTATTTTCATCTTAATTTTATAT
MT	ATTGAGATATAATAATAACATTTTTTTAAAAAATCATCTATTTTCATCTTAATTTTATAT *****
RP	ATTAAAGCCCATTTTTTGAGTATTCAAATTCAAATTATTGTGTTATTAACATCTTATATA
MT	ATTAAAGCCCATTTTTTGAGTATTCAAATTCAAATTATTGTGTTATTAACATCTTATATA *****
RP	TAAACTTTTCTATTTAATGTTAATGAAAAAGTGAATATATATACATAGAGTAATGTTATG
MT	TAAACTTTTCTATTTAATGTTAATGAAAAAGTGAATATATATACATAGAGTAATGTTATG *****
RP	ATGTATATATCAAAAAAACTAGAGGAGACGACCTTCCATGACAGAATATAAACCAACAG
MT	ATGTATATATCAAAAAAACTAGAGGAGACGACCTTCCATGACAGAATATAAACCAACAG *****
RP	TTAGATTAGCAACAAGAGATGACGTACCTAGGGCAGTAAGAACTCTTGCAGCAGCTTTTG
MT	TTAGATTAGCAACAAGAGATGACGTACCTAGGGCAGTAAGAACTCTTGCAGCAGCTTTTG *****
RP	CAGATTATCCAGCAACAAGACACACAGTTGATCCAGATAGACACATTGAAAGAGTTACAG
MT	CAGATTATCCAGCAACAAGACACACAGTTGATCCAGATAGACACATTGAAAGAGTTACAG *****
RP	AATTACAAGAATTATTTTTTAACAAGAGTTGGTCTTGATATAGGTAAAGTATGGGTGCAG
MT	AATTACAAGAATTATTTTTTAACAAGAGTTGGTCTTGATATAGGTAAAGTATGGGTGCAG *****
RP	ATGATGGAGCAGCAGTTGCAGTTTGGACAACACCAGAATCAGTTGAAGCTGGTGCTGTTT
MT	ATGATGGAGCAGCAGTTGCAGTTTGGACAACACCAGAATCAGTTGAAGCTGGTGCTGTTT *****
RP	TTGCAGAAATTGGACCAAGAATGGCTGAATTATCAGGATCAAGATTAGCAGCTCAGCAAC
MT	TTGCAGAAATTGGACCAAGAATGGCTGAATTATCAGGATCAAGATTAGCAGCTCAGCAAC *****
RP	AGATGGAAGGATTACTTGCAACACACAGACCAAAAGAACCAGCATGGTTTTAGCAACAG
MT	AGATGGAAGGATTACTTGCAACACACAGACCAAAAGAACCAGCATGGTTTTAGCAACAG *****
RP	TTGGAGTTTCACCAGATCACCAGGGAAGGTTTAGGTTCTGCTGTTGTACTTCCTGGTG
MT	TTGGAGTTTCACCAGATCACCAGGGAAGGTTTAGGTTCTGCTGTTGTACTTCCTGGTG *****
RP	TTGAAGCAGCTGAAAGAGCAGGAGTTCCAGCATTTTTAGAAACATCAGCACCAAGAAATC
MT	TTGAAGCAGCTGAAAGAGCAGGAGTTCCAGCATTTTTAGAAACATCAGCACCAAGAAATC *****
RP	TTCCATTTTATGAAAGACTTGGATTTACAGTTACAGCAGATGTTGAAGTTCCAGAAGGAC
MT	TTCCATTTTATGAAAGACTTGGATTTACAGTTACAGCAGATGTTGAAGTTCCAGAAGGAC *****
RP	CAAGAACATGGTGTATGACAAGAAAACCAGGAGCATAACCATGGCTCCGACCGAAGCCAC
MT	CAAGAACATGGTGTATGACAAGAAAACCAGGAGCATAACCATGGCTCCGACCGAAGCCAC *****
RP	CCGGGGCGGCCCGCCGACCCCGACCCGCCCCGAGGCCACCGCGGGGGACACACCGA
MT	CCGGGGCGGCCCGCCGACCCCGACCCGCCCCGAGGCCACCGCGGGGGACACACCGA *****
RP	ACACGCCGACCCTGCTGAACACGCGGCGCAGTTCGGTGCCAGGAGCGGATCGGGAATTA
MT	ACACGCCGACCCTGCTGAACACGCGGCGCAGTTCGGTGCCAGGAGCGGATCGGGAATTA

Functional characterization of a MatE transporter in *Methermicoccus shengliensis* and evolutionary diversity of archaeal MatE transporters

```

*****
RP      ATTCGAAGCTGCTGGTGAAAGAGACCCTATCTTACCTGCTAAAATCTAAGTTAATTACTA
MT      ATTCGAAGCTGCTGGTGAAAGAGACCCTATCTTACCTGCTAAAATCTAAGTTAATTACTA
*****

RP      ATTTATTATTAATTTATTATTAGATTGGGTAAAATAGTAAAAGAAAACCTAAAGGAAACCT
MT      ATTTATTATTAATTTATTATTAGATTGGGTAAAATAGTAAAAGAAAACCTAAAGGAAACCT
*****

RP      AATATGGTTTCCTTTTTTTTATATATTTTTTAATTCACCTGGGGGGATTAAAACTTCAAGGT
MT      AATATGGTTTCCTTTTTTTTATATATTTTTTAATTCACCTGGGGGGATTAAAACTTCAAGGT
*****

RP      GAGAGTATGAGGAGAGCGCGCTTTTAAACAATAACTCTCGCTTCATTCTTAACCTCCCTTC
MT      GAGAGTATGAGGAGAGCGCGCTTTTAAACAATAACTCTCGCTTCATTCTTAACCTCCCTTC
*****

RP      GCGCTTTCATCTGTAAATGTCGCTCTCCCGATGATTGGAAAAGGGTTTGCGGTGGATGCG
MT      GCGCTTTCATCTGTAAATGTCGCTCTCCCGATGATTGGAAAAGGGTTTGCGGTGGATGCG
*****

RP      ATAACGCTCAACTGGATTGCTACCGCTTATCTTCTTTCTTCCGCCATGTTTCTCATTCCA
MT      ATAACGCTCAACTGGATTGCTACCGCTTATCTTCTTTCTTCCGCCATGTTTCTCATTCCA
*****

RP      TTCGGCAGGATAGCTGATATTAAAGGAAGGAGAATGGTTTTTGTCTCGGAGTGGGTGTT
MT      TTCGGCAGGATAGCTGATATTAAAGGAAGGAGAATGGTTTTTGTCTCGGAGTGGGTGTT
*****

RP      TTTACTGCAGGATCACTGTTCTCAGGCCTATCCACTTCTGCAGAAATGCTGATAGCATTC
MT      TTTACTGCAGGATCACTGTTCTCAGGCCTATCCACTTCTGCAGAAATGCTGATAGCATTC
*****

RP      AGAGTCCTGCAGGGAATTGGAGGTGCAATGGTATTTGCGACAGGAATGGCAATTCTAATC
MT      AGAGTCCTGCAGGGAATTGGAGGTGCAATGGTATTTGCGACAGGAATGGCAATTCTAATC
*****

RP      TCCGTTTTCCCGCTAAATGAGAGGGGAAAAGTTTTAGGAATCAATGTGGCTTCAGTTTAC
MT      TCCGTTTTCCCGCTAAATGAGAGGGGAAAAGTTTTAGGAATCAATGTGGCTTCAGTTTAC
*****

RP      ACCGGCTTAAGCCTTGGCCCCCTTCTTCGGCGGCATTTTAACCCAGAACTTCGGATGGAGG
MT      ACCGGCTTAAGCCTTGGCCCCCTTCTTCGGCGGCATTTTAACCCAGAACTTCGGATGGAGG
*****

RP      AGTGTTTTTCTTGTAATGTCCCTCTCGGCATCATTGTGTGTCAGTCGCCCTATGGAAG
MT      AGTGTTTTTCTTGTAATGTCCCTCTCGGCATCATTGTGTGTCAGTCGCCCTATGGAAG
*****

RP      CTGAAAGCGGAGTGGGCTGATGCTAAAGAGGAGAGGTCGATTTGGTTGGCTCTGCAATT
MT      CTGAAAGCGGAGTGGGCTGATGCTAAAGAGGAGAGGTCGATTTGGTTGGCTCTGCAATT
*****

RP      TACAGCCTGATGCTCGTGCTGATAATGATTGGTGTGTCTGAATCAACCTGATTCTGATA
MT      TACAGCCTGATGCTCGTGCTGATAATGATTGGTGTGTCTGAATCAACCTGATTCTGATA
*****

RP      ACAGCGGGCTTGGCTCTGCTGATTGTCTTCATTGTC-----
MT      ACAGCGGGCTTGGCTCTGCTGATTGTCTTCATTGTCGGGAAAGCAGAAATCGAACATCCC
*****

RP      -----

```

MT	GTCTTGAAATAAGGCTTTTCAGGCGAAACATAACTTTTGCATTCTCAAACCTTGCGGCT
RP	-----
MT	CTGCTGAACTACGCTGCCACTTTTCGCCGTAACTACCTCTTAAGCCTATACTTGCACTAC
RP	-----
MT	ATCAAAGCTTTAACGC

Figure 3-S14. The sequencing alignment results of the $\Delta matE$ mutants. Note that we performed multiple sequence alignment using the CLUSTAL X (1.83) software. RP: recombinant plasmid, MT: mutant strain. Showed that the mutant successfully exhibited the wild-type chromosomal sequence outside both the upstream and downstream regions of the homologous recombinant plasmid.

3.6 References

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Chapter 4

**Comparative genomic insights into MatE
transporters diversity and habitat adaptation of
archaea**

Short overview of chapter IV

Chapter IV expands the investigation from a single methanogen to the archaeal domain by examining the phylogenetic, genomic, structural, and ecological diversity of MatE transporters. Large-scale comparative analyses are used to characterize their distribution across archaeal lineages, habitats, and metabolic lifestyles, while structural classification and molecular docking provide insights into their potential functional diversification. This chapter places the functional characterization of MatE in *M. shengliensis* within a broader evolutionary and ecological context.

4.1 Abstract

Archaea comprise deeply rooted and phylogenetically diverse lineages that inhabit a wide range of environments and play essential roles in global biogeochemical cycles. However, the diversity of MATE (Multidrug and Toxic Compound Extrusion) family transporters in archaea, which are presumably involved in habitat adaptation, remains poorly understood. Here, we systematically analyzed archaeal MatE transporters using large-scale phylogenetic and comparative genomic analyses, combined with structure-based clustering and molecular docking. Our results show that MatE transporters are significantly enriched in archaea from host-associated and hypersaline environments compared with those from other habitats. Specific MatE transporters are strongly associated with particular habitats, and their copy numbers are positively correlated with genome size. Moreover, MatE transporters in archaea exhibit structural diversity and can be classified into four structural classes, among which Class I is predominant in both abundance and phylogenetic distribution compared with Classes II, III, and IV. Overall, these findings indicate that the successful adaptation of archaea to specific habitats is related to the acquisition and maintenance of MatE transporters, which may be critical for their survival in these environments.

Keywords: Archaea, MatE transporter, Habitat adaptation, Metabolic type

4.2 Introduction

Archaea represent one of the most ancient and deeply rooted lineages of life and exhibit remarkable diversity in metabolic strategies, cellular physiology, and ecological distribution (Bontognali et al., 2012; Spang et al., 2017). Early studies primarily associated archaea with extreme environments, such as highly acidic, saline, or high-temperature habitats (Woese et al., 1978), which strongly shaped the traditional view of archaea as extremophiles. However, with the rapid development of culture-independent approaches and genome-resolved metagenomics (Bornemann et al., 2022; He et al., 2021), it has become increasingly evident that archaea are not confined to extreme niches but are instead widely distributed across a broad range of natural and engineered environments. These include host-associated, hypersaline, freshwater, bioreactor, terrestrial, hydrothermal vent, marine, hot spring, cold spring, and oil field environments (Borrel et al., 2020; Chaudhary et al., 2009; Liang et al., 2018; Sriaporn et al., 2023; Wang et al., 2020). This extensive ecological distribution

suggests that archaea have evolved diverse and flexible molecular mechanisms to cope with complex and fluctuating environmental conditions.

Among the cellular systems that underpin archaeal environmental adaptability, membrane transport systems play a particularly critical role. Membrane transporters are essential for maintaining cellular homeostasis, facilitating nutrient uptake, eliminating toxic compounds, and regulating osmotic balance (Omote et al., 2006; Vujica et al., 2023), thereby enabling microorganisms to survive in environments with highly variable chemical and physical properties. The MATE (Multidrug and Toxic Compound Extrusion) family comprises a group of integral membrane proteins that are widely distributed across prokaryotes and eukaryotes (Moriyama et al., 2008; Upadhyay et al., 2019). Members of this family have been implicated in a broad range of biological functions, including the extrusion of xenobiotics and toxic compounds, accumulation of secondary metabolites, detoxification of herbicides, translocation of metal ions such as Fe^{2+} , hormone signaling, aluminum (Al) transport, and participation in host-pathogen interactions (Du et al., 2021; Ghanizadeh et al., 2020; Nawrath et al., 2002; Omote et al., 2006; Tanaka et al., 2021; Wang et al., 2016). Despite their functional importance in other domains of life, MatE transporters in archaea remain poorly characterized, and their diversity, evolutionary distribution, and potential roles in environmental adaptation have not yet been systematically investigated.

In our previous study, we demonstrated that the methanogenic archaeon *Methermicoccus shengliensis* employs MATE family transporters to mediate the uptake of methoxylated aromatic compounds from the extracellular environment, thereby establishing a direct link between MatE transporters and archaeal energy metabolism and methanogenesis (Leng et al., 2025). This finding provides experimental evidence that MatE transporters can play functionally significant roles in archaeal physiology. However, whether such transporters are broadly distributed across archaeal lineages, how their occurrence varies among different ecological niches, and whether they exhibit diversity remain open questions.

In this study, we performed large-scale phylogenetic and comparative genomic analyses to investigate the distribution and diversity of MatE transporters across archaea. We show that MatE transporters are widely distributed among archaea and are significantly enriched in host-associated and hypersaline environments, occurring predominantly in heterotrophic, hydrogenotrophic, and methylophilic archaea. Notably, the copy number of MatE transporters is positively correlated with genome

size, suggesting that their expansion is jointly shaped by genome complexity and ecological selection. Furthermore, by integrating protein structural-based clustering and molecular docking analyses, we reveal clear structural diversity among archaeal MatE transporters, which can be classified into four distinct structural classes. These classes exhibit differences in key substrate-binding residues when interacting with identical substrates, providing structural insights into the potential functional diversification of MatE transporters. Collectively, our results highlight the contribution of MatE transporter diversity to archaeal environmental adaptation and metabolic flexibility.

4.3 Material and Methods

4.3.1 Genome datasets

The sequences of archaea genomes were downloaded from NCBI (<https://www.ncbi.nlm.nih.gov/genome/microbes/>, accessed on 15 January 2024), JGI (<https://img.jgi.doe.gov/>, accessed on 15 January 2024), EBI-gut-rumen (<https://www.ebi.ac.uk/metagenomics/genome-catalogues/human-gut-v2-0-2>, accessed on 15 January 2024), and GTDB (<https://gtdb.ecogenomic.org/>, accessed on 15 January 2024). A total of 10,864 genomes were collected for downstream analyses. To obtain a more reliable genome dataset, we assessed the quality of each genome using CheckM2 (version 1.0.2) (Chklovski et al., 2023), which determines the estimated completeness of a genome and detects possible contamination based on lineage-specific sets of single-copy genes. We further compiled a high-quality dataset, including only genomes that were nearly complete (completeness $\geq 90\%$) and had low contamination (less than 5% contamination). All 10,864 genomes were subjected to quality control according to these criteria to reduce data redundancy and biased genome representation of Archaea.

4.3.2 Collection of metadata

A variety of habitats were included in the analysis. Habitats of the 10864 archaea strains were derived from their isolation source. The isolation source for each strain was determined manually by searching IMG metadata (<https://img.jgi.doe.gov/>, accessed on 18 May 2024), NCBI Biosample (<https://www.ncbi.nlm.nih.gov/biosample/?term=>, accessed on 18 May 2024), GTDB (<https://gtdb.ecogenomic.org/>, accessed on 18 May 2024), ATCC (<https://www.atcc.org/>, accessed on 18 May 2024), and the scientific literature. Based

on their isolation sources, we categorized the genomes into host-associated, hypersaline environments, freshwater, bioreactor, terrestrial, hydrothermal vent, marine, hot spring, cold spring, and oil field; genomes whose source of isolation was not among these major habitats were labeled as belonging to other habitat; and genomes without isolation information were labeled as belonging to unknown habitat.

4.3.3 Bioinformatics and data analysis software

Phylogenetic trees were constructed using IQ-TREE2 (version 2.2.2.6) (Minh et al., 2020). Homologous protein searches were performed using BLAST 2.15.0 against local genomes (Camacho et al., 2009), and multiple sequence alignments (MSAs) were generated using MAFFT v7.526 with default parameters (Rozewicki et al., 2019). Protein topologies and domain architectures were predicted using SMART (<https://smart.embl.de/>, accessed on 23 June 2024) (Letunic et al., 2021), CDvist (<http://cdvist.utk.edu/>, accessed on 23 June 2024) (Adebali et al., 2015), and HHpred (<https://toolkit.tuebingen.mpg.de/tools/hhpred>, accessed on 23 June 2024) (Gabler et al., 2020). Phylogenetic trees were visualized and annotated using iTOL (<http://itol.embl.de/>, accessed on 16 December 2024).

4.3.4 Identification and analysis of protein

Genes encoding MatE transporters in the selected genomes were identified by BLASTP, with hits showing an E-value $< 1 \times 10^{-5}$ considered as candidates. The protein sequence of the MatE domains of transporters from *Methermicoccus shengliensis* DSM_18856 (BP07_RS03225) (Leng et al., 2025) were selected as query sequences to perform the BLASTP analysis. All candidate proteins were manually reexamined for domain organization using SMART, HHpred, and CDvist (Adebali et al., 2015; Gabler et al., 2020; Letunic et al., 2021). A complete list of identified MATE transporter proteins is provided in Table S2.

4.3.5 Phylogenetic analysis

To construct a phylogenetic tree from the 10864 archaeal genomes in our Lab dataset (<https://doi.org/10.5281/zenodo.18079802>, accessed on 29 December 2025 and **Table 4-S1**) multiple sequence alignments were generated by concatenating 122 phylogenetically informative protein-or protein domain-encoding sequences from the Pfam v27 (Mistry et al., 2021) and TIGRFAMs v15.0 databases (Haft et al., 2003). These 122 archaeal marker proteins were selected following previously established

criteria (Cha et al., 2022). The phylogenetic tree was constructed using IQ-TREE2 (Minh et al., 2020) (-s example.phy -m MFP -B 1000 --bnni -T AUTO), and bootstrap analysis was applied with 1,000 replications.

4.3.6 AlphaFold3 Prediction, Structure Comparison and Docking Analysis

The three-dimensional (3D) structure of the MatE transporter from we previously reported *M. shengliensis* strain was selected as a structural reference (Leng et al., 2025), and structurally aligned with the experimentally resolved archaeal MATE transporter PfMATE from *Pyrococcus furiosus* (PDB ID: 3VVN). Structural deviation was quantified by calculating the root mean square deviation (RMSD), yielding a value of 0.713 Å. The 3D structures of 853 MatE homologs from representative strains were predicted using AlphaFold3 (<https://alphafoldserver.com/>, accessed on 15 January 2025). The quality of predicted structures was evaluated using pLDDT scores, which indicated high overall confidence (average score > 90) (Meng et al., 2025). All Structural visualizations were generated using PyMOL (<http://www.pymol.org/>, accessed on 24 March 2025). Small-molecule ligands were obtained from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>, accessed on 26 May 2025) and preprocessed using Molecular Operating Environment (MOE) version 2022.02 to generate appropriate conformations and isomers (Vilar et al., 2008). The processed ligands were saved in the ligand database in “.pdb” format. Receptor protein structures were obtained from AlphaFold (<https://alphafoldserver.com/>, accessed on 15 January 2025), further refined using the QuickPrep module in MOE, and their active sites were identified using the Site Finder tool (Frimayanti et al., 2025). The triangular matcher method was used for ligand placement, with London dG applied during this stage. An Induced Fit model was employed for refinement, utilizing GBVI/WSA dG in this phase (Guo et al., 2025). The placement and refinement steps were set to generate 50 and 20 positions, respectively, before initiating the docking process.

4.3.7 Statistical Analysis

All statistical analyses and graphical visualizations were performed using GraphPad Prism 10 (GraphPad Software, San Diego, CA, USA). One-way analysis of variance (ANOVA) with Welch’s correction was applied for all group comparisons to account for unequal variances. Data are presented as mean ± standard deviation unless otherwise stated. A *p* value < 0.05 was considered statistically significant.

4.4 Results and Discussion

4.4.1 Diversity of MatE transporters in archaea

To systematically examine the diversity of MatE transporters in archaea, we performed a large-scale comparative genomic analysis based on a well-annotated archaeal phylogenetic framework comprising 10,864 genomes spanning four major superphyla: Asgard, TACK (*Thaumarchaeota*, *Aigarchaeota*, *Crenarchaeota*, and *Korarchaeota*), DPANN (*Diapherotrites*, *Parvarchaeota*, *Aenigmarchaeota*, *Nanohaloarchaeota*, and *Nanoarchaeota*), and *Euryarchaeota* (Spang et al., 2017). Among the analyzed genomes, 4,351 archaeal strains encode at least one MatE transporter, whereas 6,513 genomes lack detectable MatE homologs (**Figure 4-1 and 4-S1**). This uneven distribution indicates substantial variability in MatE presence across archaeal lineages. In parallel, we observed notable differences in genome size across environmental categories. For example, archaeal genomes from hypersaline environments tend to be relatively larger (0.37–7.28 Mb, with most around 2.8 Mb), whereas genomes from freshwater-associated archaea are generally smaller (0.29–5.17 Mb, with most around 1.2 Mb). These patterns suggest that genome architecture varies considerably among archaeal populations occupying distinct ecological niches. In addition, we also observed an association between genomic GC content and phylogenetic placement: closely related strains tend to exhibit similar GC contents (**Figure 4-1**). Archaeal lineages sharing similar habitats and metabolic features tend to cluster locally within the phylogenetic tree, separated by small numbers of branches, but they are relatively scattered in terms of overall evolutionary relationships. Thus, patterns of ecological distribution, genome architecture, and metabolic potential in archaea cannot be fully explained by phylogenetic relationships alone, suggesting that factors beyond phylogenetic tree contribute to archaeal ecological diversification.

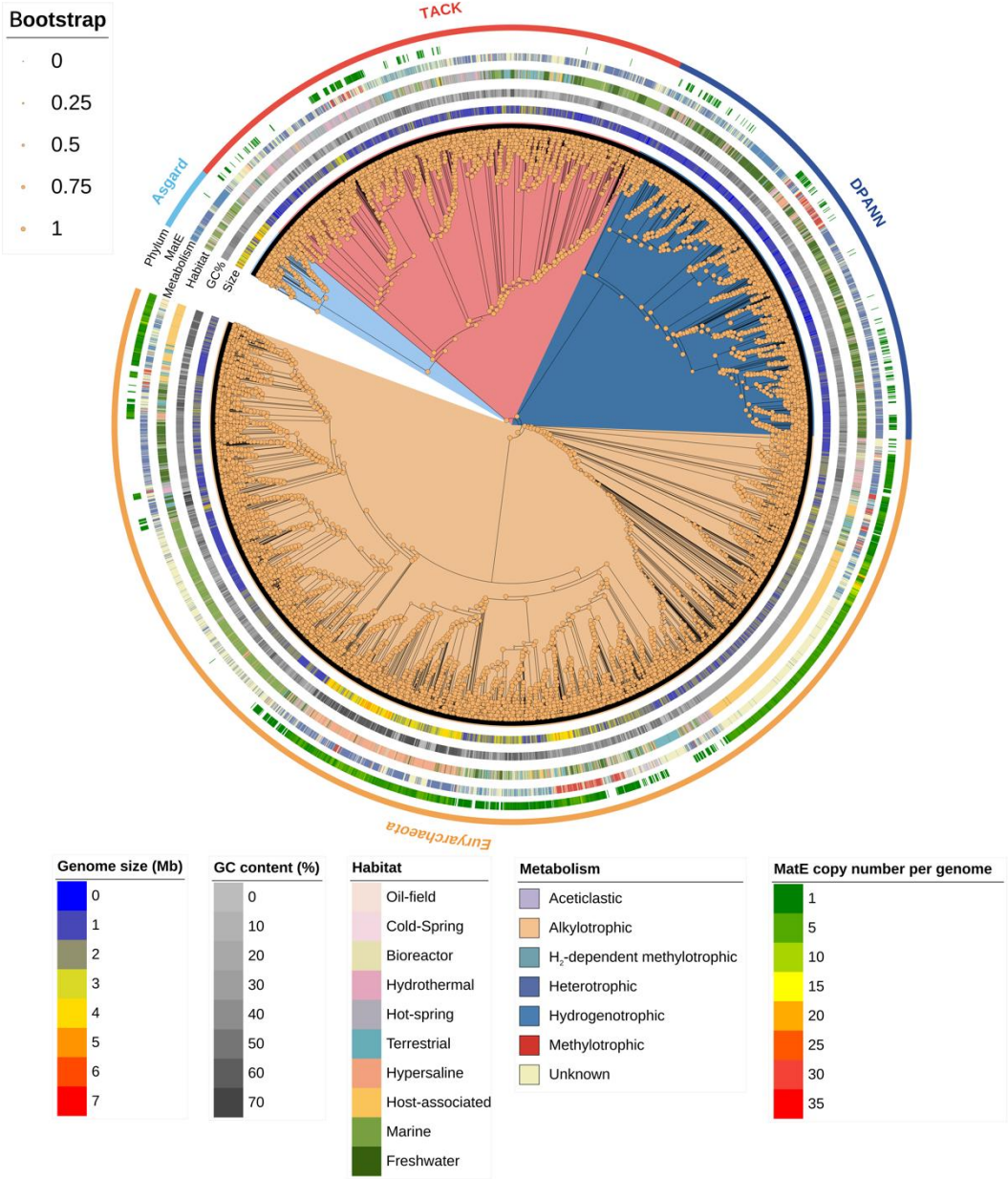


Figure 4-1. Phylogenetic tree of archaea. A maximum-likelihood phylogenetic tree was constructed based on 10,864 archaeal genomes (see **Table 4-S1**). The outer rings indicate genome size (Mb), GC content (%), habitat categories, metabolic types, and the copy number of MatE-encoding genes per genome, based on a total of 4,351 MatE proteins identified across the dataset. Blue, red, navy blue, and orange denote members of the Asgard, TACK, DPANN, and *Euryarchaeota* supergroups, respectively. Genomes with unassigned metabolic features are categorized as Unknown. Bootstrap support values were calculated from 1,000 replicate trees and are indicated by node color intensity. Black lines represent phylogenetic branches.

4.4.2 Enriched MatE transporter in a specific habitat

To further investigate the ecological distribution of MatE transporters in archaea, we systematically integrated the niche information and metabolic type annotation for all analyzed genomes. Based on the available metadata, archaeal strains were classified into ten habitat types and six metabolic categories. To control for potential habitat- or metabolism-driven biases, genomes encoding MatE transporters were compared with genomes lacking MatE homologs within the same ecological environment and metabolic categories. Across habitats, MatE transporters exhibited a non-random distribution. They were significantly more abundant in host-associated and hypersaline environments than in other habitats, whereas genomes from oil-field and cold-spring environments showed markedly lower abundance, indicating a clear environmental preference (**Figure 4-2A**). A similar pattern was observed across metabolic types: MatE transporters were mainly enriched in heterotrophic, hydrogenotrophic, and methylotrophic archaea, but were substantially less abundant in H₂-dependent methylotrophic, acetoclastic, and alkylotrophic archaea (**Figure 4-2B**). These results suggest that MatE expansion may be linked to specific energy metabolism strategies, particularly those involving diverse organic substrates.

To evaluate whether these patterns could be explained solely by habitat or metabolic composition, we further compared genome size and GC content between MatE-lacking and MatE-containing genomes under identical ecological and metabolic stratifications (**Figure 4-2C-F** and **Figure 4-S2**). MatE-lacking genomes were primarily found in marine and freshwater environments and among heterotrophic archaea. These genomes were relatively compact, with median sizes of approximately 1.2 Mb in marine environments, 1.4 Mb in freshwater environments, and 1.2 Mb in heterotrophic archaea, although maximum sizes reached up to 6.6 Mb (**Figure 4-2C and 4-2D**). Their GC content showed a narrow distribution, clustering around ~40% (**Figure 4-S2A and 4-S2B**). In contrast, MatE-containing genomes displayed distinct genomic features under the same ecological and metabolic classifications. These genomes were primarily distributed in host-associated and hypersaline environments and among heterotrophic archaea, with substantially larger median genome sizes of approximately 1.9 Mb, 3.1 Mb, and 2.8 Mb, respectively, and maximum sizes approaching 7.5 Mb (**Figure 4-2E and 4-2F**). GC content also varied across habitats, with median values of approximately 32% in host-associated environments and 65% and 62% in hypersaline environments and heterotrophic archaea, respectively (**Figure 4-S2C and 4-S2D**). Overall, these results indicate that the distribution of MatE transporters in archaea is non-random and consistently associated with specific habitats and metabolic types, reflecting coordinated patterns with genome size and GC content.

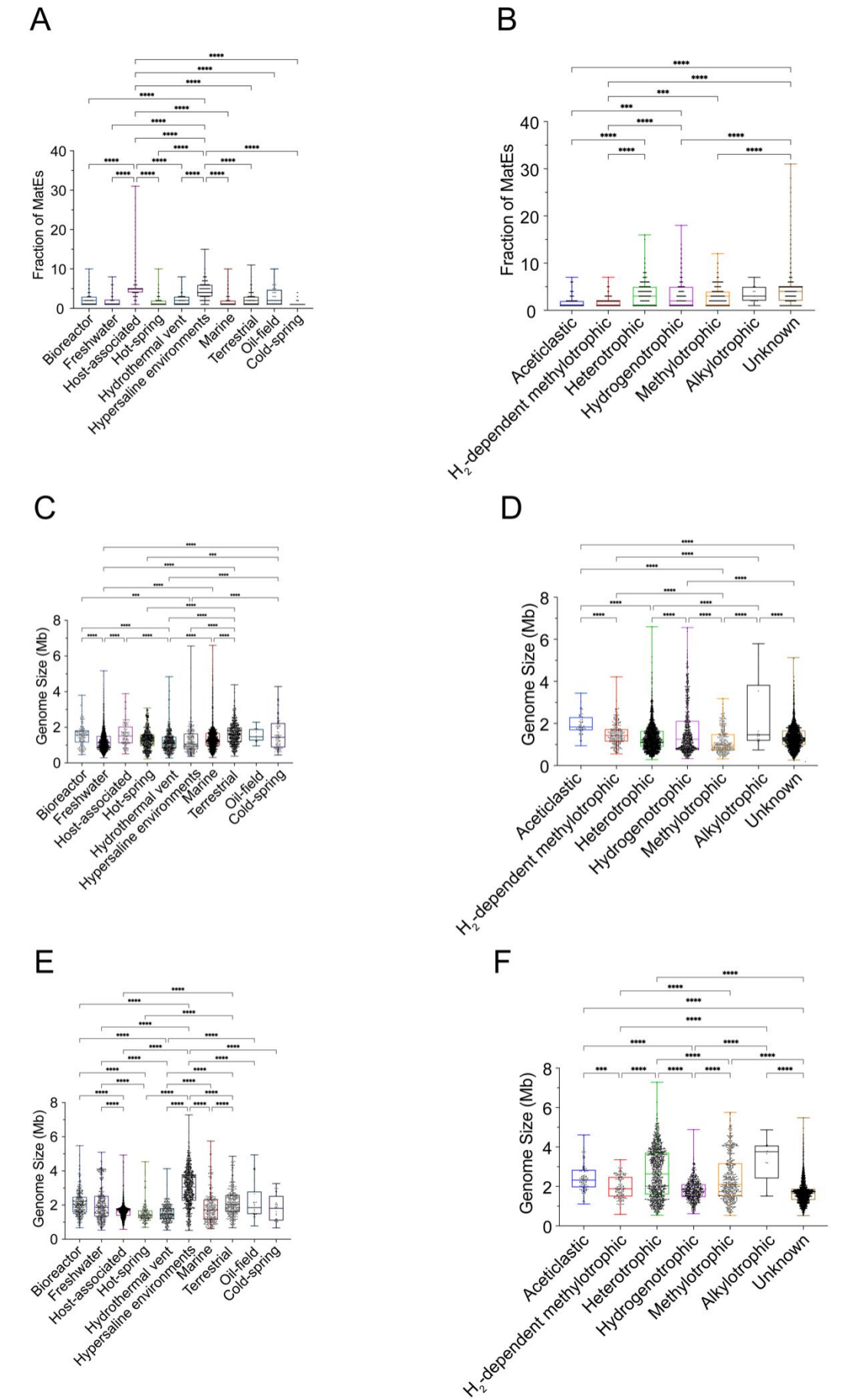


Figure 4-2. MatE transporters are associated with archaeal habitats and metabolic strategies. **A.** Abundance of MatE transporters across different habitats among 4,351 archaeal genomes containing MatE. **B.** Abundance of MatE transporters across different archaeal metabolic types among the same 4,351 MatE-containing genomes. Alkylotrophic and heterotrophic are used as descriptive categories based on genome annotations or literature evidence, whereas H₂-dependent methylotrophic, acetoclastic, hydrogenotrophic, and methylotrophic metabolisms represent canonical archaeal metabolic pathways. Genomes with unassigned metabolic features are categorized as Unknown. **C.** Relationship between habitats and genome size in 6,513 archaeal genomes lacking MatE transporters. **D.** Relationship between metabolic types and genome size in the same 6,513 MatE-lacking genomes. **E.** Relationship between habitats and genome size in 4,351 MatE-containing archaeal genomes. **F.** Relationship between metabolic types and genome size in the same 4,351 MatE-containing genomes. Panels **C**, **D** serve as control groups (genomes lacking MatE), whereas panels **E**, **F** represent experimental groups (genomes containing MatE). Colors indicate different habitat categories or metabolic types as labeled on the x-axis. In each boxplot, the median is shown in the box as a thick bar (unless it coincides with the borderline). The 25th and 75th percentiles are, respectively, in the lower and upper bounds of the box. Lines through the boxes indicate the minimum value and maximum values, while the whiskers correspond to the 1.5 interquartile range from the bounds. Asterisks indicate levels of statistical significance determined by one-way ANOVA with Welch's correction (***, $p < 0.001$; ****, $p < 0.0001$).

4.4.3 Relationship between genome size and MatE abundance

To assess whether the uneven distribution of MatE transporters in archaeal genomes can be attributed to general genome traits, we further analyzed the relationship between genome size and the abundance of MatE transporters. The results showed that genomes lacking MatE were distributed over the entire range genome sizes range, and their MatE abundance was 0 (**Figure 4-3**). The average genome size of the strains containing MatE was 2.04 Mb, which was significantly higher than that of the strains without MatE (**Figure 4-3**). The results indicate that the presence of MatE transporters is related to the genome size of archaeal, and their uneven distribution is not random but tends to occur in strains with larger genomes.

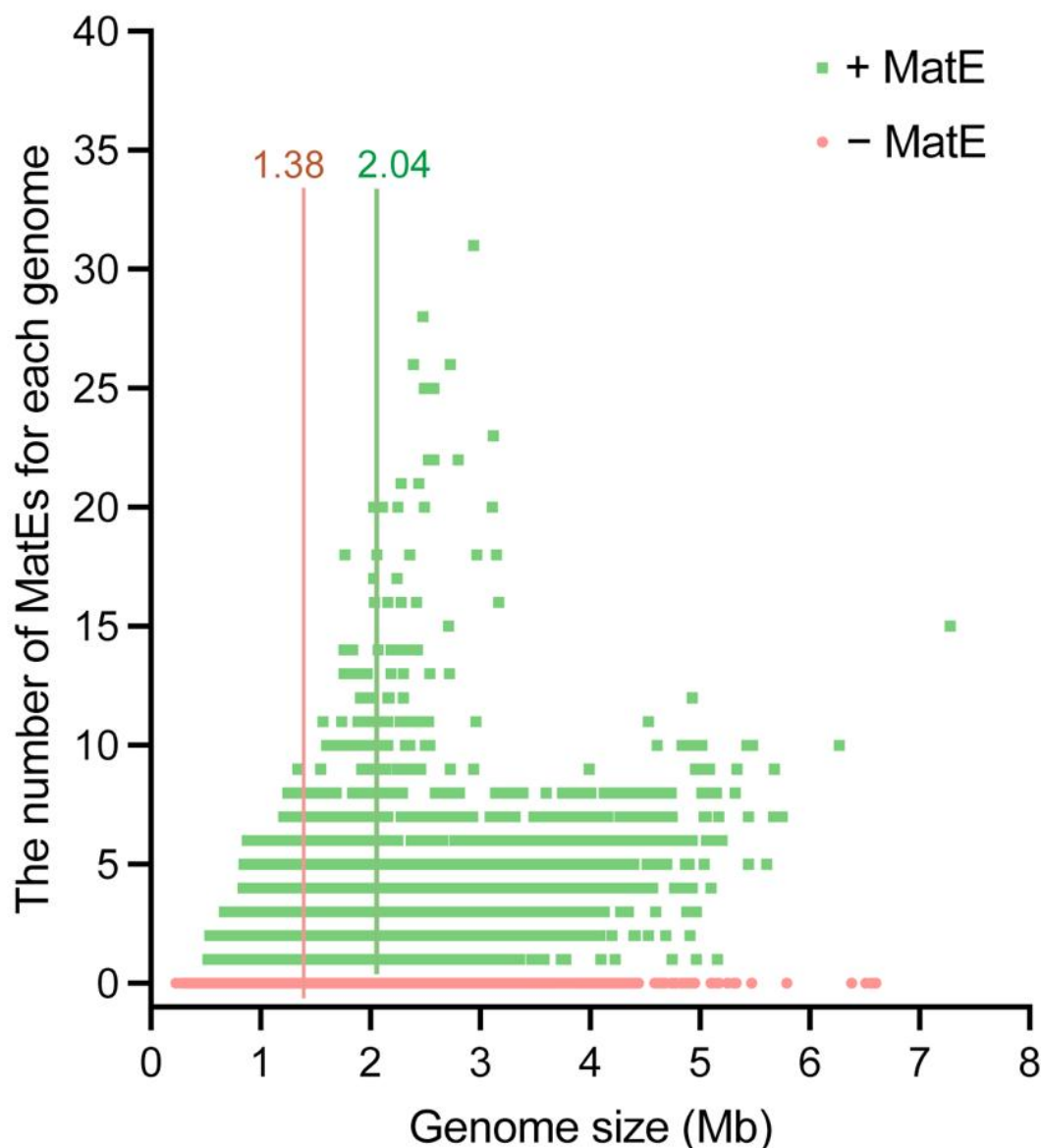


Figure 4-3. Correlation between MatE number and genome size. Relationship between the number of MatEs and the genome size, which varies from 0.23 to 7.28 Mb. The green dots represent strains with MatEs, and the green line represents their median number; the red dots represent strains that lack MatEs, and the red line represents their median number. “+ MatE” and “– MatE” indicate strains that do and do not contain MatE, respectively. Each point represents a single archaeal genome. Genomes lacking MatE transporters are shown at zero abundance.

4.4.4 Variations in habitats account for the structural diversity of MatE

To study the evolutionary distribution and structural diversity of archaeal MatE transporters, we used phylogenetic analysis and in combination with protein structure prediction using AlphaFold3 (<https://alphafoldserver.com/>, accessed on 15 January 2025) (Abramson et al., 2024). Archaea encoding MatE transporters were divided into four superphyla: Asgard (2 strains), TACK (264 strains), DPANN (277 strains), and *Euryarchaeota* (3,808 strains) (**Figure 4-4A**). A total of 853 representative MatE

proteins were selected from the above groups (**Table 4-S3**) and compared with the structure of MatE from *M. shengliensis*. Based on structural clustering analysis, archaeal MatE transporters were classified into four distinct structural classes, designated Class I, Class II, Class III and Class IV (**Figure 4-4B**). Phylogenetic analysis showed that Class I MatE transporters are mainly distributed in TACK, *Euryarchaeota*, and DPANN superphyla, occupying a major proportion in different habitats and metabolic types. In contrast, Classes II, III, and IV members were distributed in multiple phylogenetic branches, but were less abundant in all habitats and metabolic types (**Figure 4-4 and 4-S3**). Moreover, functional prediction annotations show that the Class I MatE transporters are primarily annotated as MATE family efflux transporter, whereas Class II, Class III, and Class IV are annotated as oligosaccharide flippase family protein, polysaccharide biosynthesis protein, and flippase, respectively (**Table 4-S3**). Taken together, these results show that archaeal MatE transporters are dominated by a single, widely distributed structural class (Class I), which accounts for approximately 99% of the analyzed proteins, whereas Classes II-IV are rare and sporadically distributed.

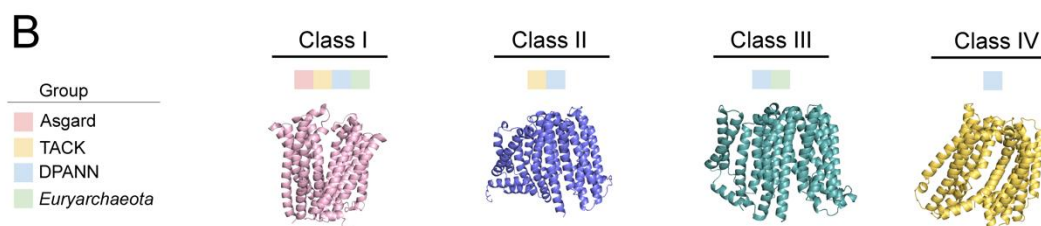
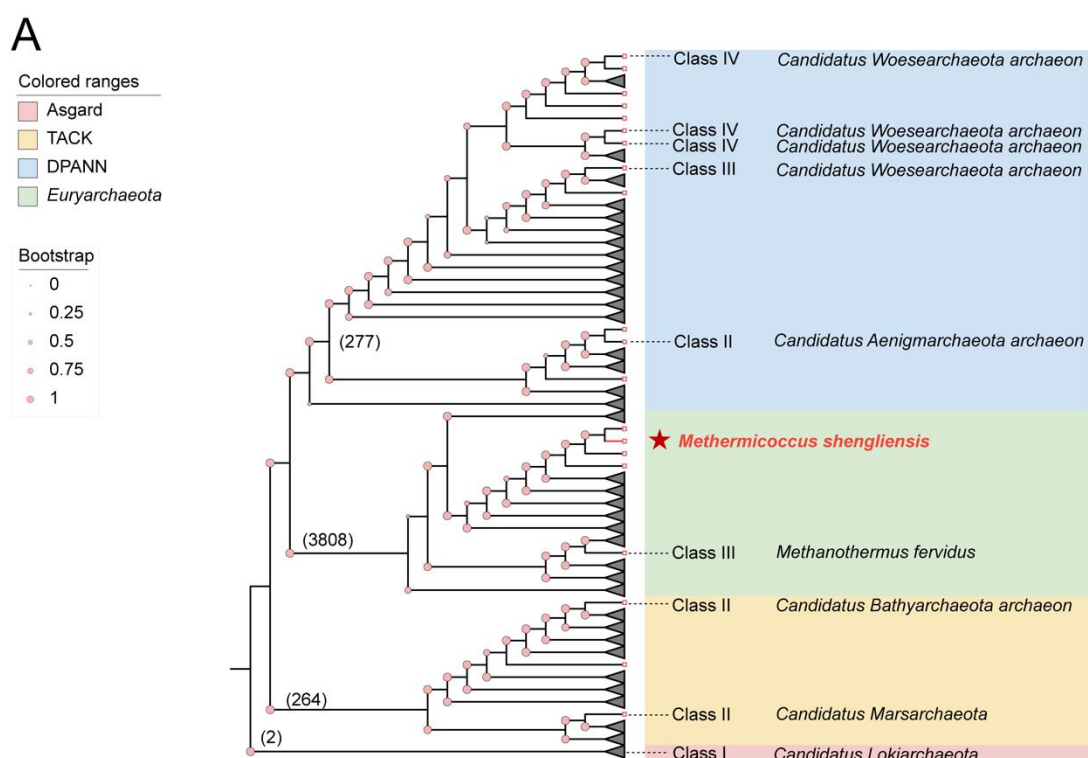


Figure 4-4. Diversity and distribution of MatE transporters. **A.** Phylogenetic tree of MatE proteins. The numbers in parentheses indicate the number of archaeal MatE proteins identified in each superphylum (Asgard, TACK, DPANN and *Euryarchaeota*). Based on structural configurations, MatE transporters are classified into four types (Class I to Class IV), with unannotated types considered as Class I. The phylogenetic tree was constructed using the maximum likelihood method with 1,000 bootstrap replicates. Tree visualization was performed using iTOL (<https://itol.embl.de/>, accessed on 12 June 2025). **B.** Overall structures of different MatE proteins.

4.4.5 MatE transporters recognize and interact with diverse substrates through specific amino acid residues

In order to analyze the interaction between different structural classes of archaeal MatE transporters and various substrates, molecular docking analyses were performed. Representative structures of MatE Classes I-IV were docked with a variety of potential substrates, including methanol, methylamine, dimethyl sulfide, 1,2,3-trimethoxybenzene, 2-methoxybenzoate, and 3,4,5-trimethoxybenzoate (**Figure 4-5 and Table 4-S4**). For substrate molecules such as methanol, methylamine, and dimethyl sulfide, stable docking conformations were not obtained, which may be related to their small molecular weight and the difficulty of forming stable interactions in the predicted binding cavity. In contrast, the larger methoxylated aromatic compounds exhibited a recognizable binding mode in all four classes of MatE (**Figure 4-5**). Notably, there is a specific residue in each class of MatE structure that consistently participates in the interactions with three methoxylated aromatic compounds: R²⁰⁸ in Class I, T⁴⁰³ in Class II, R¹⁵⁸ in Class III, and Y¹⁵⁶ in Class IV (**Figure 4-5**). To further evaluate whether other types of substrates exhibit similar interaction patterns with the MatE transporters, antibiotic norfloxacin and polysaccharide raffinose were selected for molecular docking analysis with four classes of MatE structures, respectively (**Figure 4-S4**). In Class I transporters, residues W²⁵⁸ and K⁴³⁵ were found to interact with both norfloxacin and raffinose. In Classes II and III, the residues T⁴⁰³ and R¹⁵⁸, previously involved in binding methoxylated aromatic compounds, were also involved in raffinose docking (**Figure 4-5 and 4-S4**). However, in Class IV, the residues interacting with norfloxacin (R¹⁴⁵) and raffinose (F⁵⁰ and E⁶³) did not overlap with those involved in methoxylated aromatic compound binding (**Figure 4-S4**). Taken together, these molecular docking

results indicate that archaeal MatE transporters with different structural classes have significant differences in the predicted interaction patterns of substrates and residues.

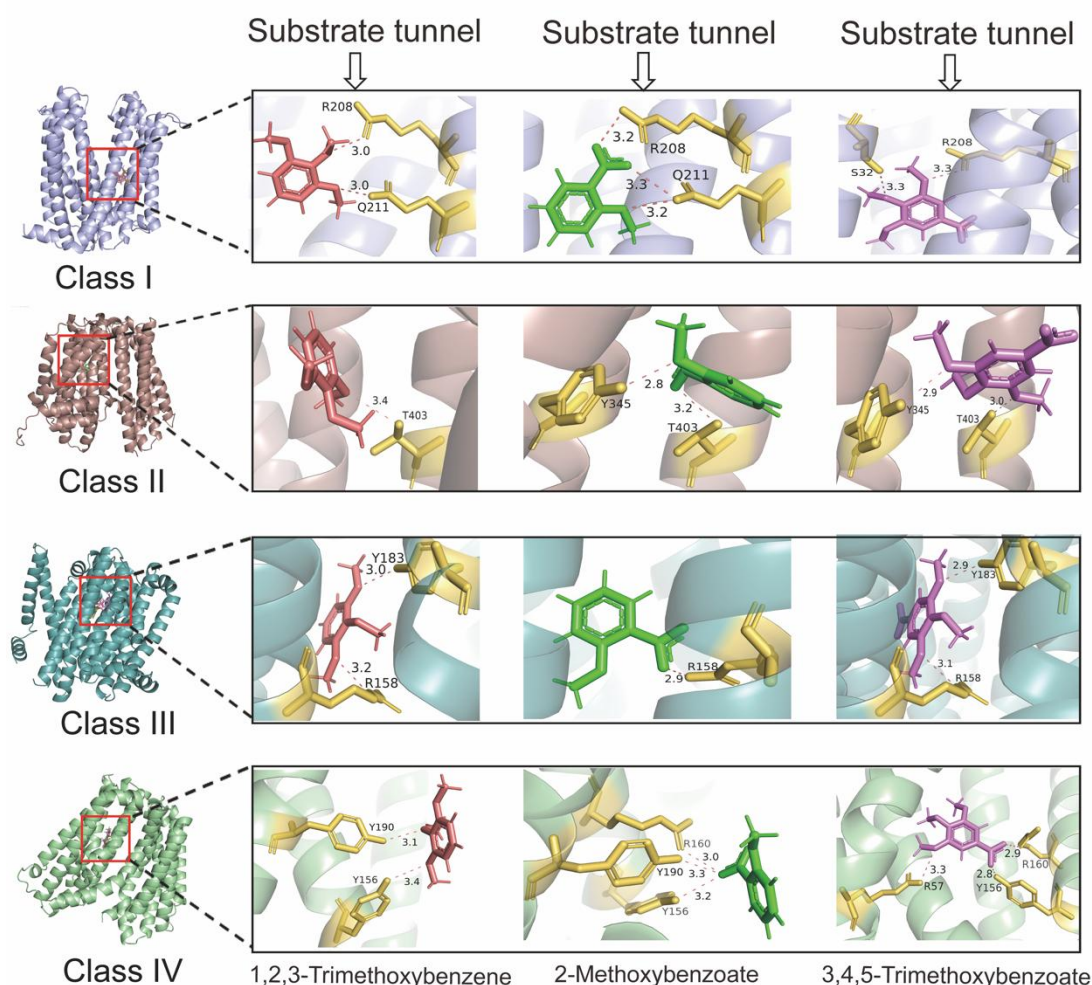


Figure 4-5. Molecular docking of MatE transporters with different substrates. The figure shows the key residues at the active sites of Class I, Class II, Class III, and Class IV MatE structures when interacting with the substrates 1,2,3-Trimethoxybenzene, 2-Methoxybenzoate, and 3,4,5-Trimethoxybenzoate. Protein backbones are shown in cartoon representation and colored differently for each class. Substrates are shown as stick models and colored red (1,2,3-trimethoxybenzene), green (2-methoxybenzoate), and purple (3,4,5-trimethoxybenzoate). Key interacting residues are shown as yellow sticks. Red dashed lines indicate hydrogen bonds, and the numbers next to the dashed lines represent the distances between atoms or residues in the protein, measured in Ångströms (Å).

4.5 Supplementary files

Table 4-S1. Taxonomic annotations were obtained from the source databases (NCBI, GTDB, JGI and EBI-gut-rumen), and differences between classification systems may exist.

Table 4-S2. Phylogenetic tree of MatE proteins.

Table 4-S3. Results of structural clustering of MatE proteins.

Note: Due to the large size of **Tables 4 S1–S3**, they will not be included in the paper. Please refer to the attached Excel file.

Table 4-S4. Information on representative MatE proteins from different structural clusters.

Group	Organism Name	Protein ID	Structural Class	Sequences	Length (aa)	Potential Substrates	References
Asgard	<i>Candidatus Lokiarchaeota archaeon bin106</i>	MBY9005203.1	Class I	MKQIFKVL TDNKDFY PRLYKIAIP IIIDFLISSL VNLLDVF MVGKLGE PALAAVGI GSRFFFLSI LLMVGIGS GAAVFTA QYWGKKD IKKIKPILG ISLTLTTIA SIIFSLISLI LPAEIMGL FTNDPIVIN LGIDYIRIL GIGQIFFGI SISFFSILTS TENVKFT MSSTALAL IFDTIISYL LIFGIGIFP ELGVIGVA IGTFFGRFF QLILILFFT YFFKLPA GKLKELFS FSWSLFRK YLDKAIPV ILQHVIWG LGSIMFVY VYAQLSTE SIAAYQAA ATIEEICLL FFTGIGISA SIMIGNRIG ANEEKKSI NYSEKFLV IYILSFIIM	455	Amino acids and/or peptides	(Imachi et al., 2020)

Functional characterization of a MatE transporter in *Methermicoccus shengliensis* and evolutionary diversity of archaeal MatE transporters

				LILLVKDI VAGLFNV SPLSKSYIS SIIFVLSIAI WGKASNI LFYTGILK GGGDTRF CMILDIGG MWLIGVPI AFIAAFTF NFPIHIIAL ISIDEFIKM IIGYKRFLS RKWIHNL TEK			
TACK	<i>Candidatus</i> <i>Marsarchaeota</i> <i>G2</i> <i>archaeon</i> <i>ECH_B_S</i> <i>AG-M15</i>	PSN92 178.1	Class II	MLKQRAA SGAGHLF VGNTLSQI IQGIGILV ARLLGAT NYGLYTL LLVPSATF LVFTQVGF GGAATRY VAYYNAL GKRAEAD SIATSTFLY ATMLYAFI SFISAVVA PAYASYV LHQPTIAY LAQLASV MIFFQGLM NYTTAVL NGYYKTK QVSAIMLI QSVAKTSL SIGLILIGL RIYGALIG TIISFVIPAI VGTIWMF NATQTKKI NFDAKTF KTITSFGLP	521	Chemoorg anotrophs	(Jay et al., 2018)

				LYAANILN GISRQIQL VILASFAS AAAVGAF TATQNLA TLISVVVY PVSTMAG VAFSEVSA QNNKARI NSSYVSAT KIATILVV PISIFMML TAKPIVTIL YSSAYS RF YLLLSIFA AGYLSVG LGGQTQTP LFAGLNNT KLNTYSA AISAVSLL SLSYGLA MGLNVFG VALATTIS TITTA AVI HIVIVRGL GVKLPKA KLVAVYL AGALSAA PLILIPQKI FRHGLIGL LELALILS VYIIISTV LPLVRGV NKDELALI SHSLKGVP ILGDILAA LTKYSKM FIHEHTEK PS			
DPAN N	<i>Candidatus</i> <i>Woesearchaeota</i> <i>archaeon</i>	DAGG 010000 29.1	Class III	MSYTKKA FWSFSVIFI CLALANV FG YLLRLV IARSLTPA	490	Methyl compound s	(Peng et al., 2022)

Functional characterization of a MatE transporter in *Methermicoccus shengliensis* and evolutionary diversity of archaeal MatE transporters

	<i>UBA525</i>			EYGLIYAV MALLSFFG FAQTLGLS DALVNAIP GIRD LKR MKSALITV IYTQLIVT GTILLIVLL LARWLAA EYFGTMV AVPLLILT AIMVFISPI EVSLIAFF QGKGLTR WYGYLTL LRAVVLL AIVVALLA LGFGVFA VMLGYVL MYLVIPVV YIPLMRKA FPEYWRV KARFDPTL LRRFIAFG FPIILASGI GFFLT YID TFFLT LFR TLPEVGLY NAALPTA GLLWFVG GVIYAVM FPLSSEM Q AKKGAGV LTDGIGRM YLYLSILL FPFAAII VI YPGIILNLL FGGAYVG AALALQIL VIGGVFYT FATLNSSII AGLGHPR KALYGLV AA AVVNV			
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				IGDILFIPR YGIAGSAA VTLVAFIV LLAVHTV VLRKIVPY GIPGLRMA GVLLATA GAVGVVF LVRLTELT MWWKIGV GVLVAGI MYAVLLF ALRVITVK ELKDLKT MIGR			
DPAN N	<i>Candidatus</i> <i>Woesearchaeota</i> <i>UBA10107</i>	HIH39 518.1	Class I V	MRLSKLF KDVAWTS VVFVFGAF LGYGMRL FARNLSSS DYGLFYAI VSFFALFS LFRSLGMT ESLVHFIP KYVAMQS WNKLRLGL IRFVFSIQL PLSFIFSL FFIFAKSIA VNFFHLES AAPLIRLQ AITFFIIGV VEILVSIFR GFQNPIFA SLYDPIRL FIMVAASV MFLKLIKIF HIDNLFLA WLLSYLV LAVLYFIQ LYLKYKKI FSFSIKKE KSVVKDI MSYSIPLM	496	Methyl compound s	(Peng et al., 2022)

Functional characterization of a MatE transporter in *Methermicoccus shengliensis* and evolutionary diversity of archaeal MatE transporters

				LGVGVQII FARIDELIL LFFKGTEQ VALYEIAY PASQLMMI LVSPFLFIL LPSISKLYF EKNKEGIR NILQMIYN AGLFFAAP LALLLFFY PEIIIKLLFS SKYLVAST ALQVMTF GTFFLMFS NINLNVLS GMGKIAT RTKILYVI GIFNIVFNL ILVPKFGY MGAVFAT SSAFFLLW FSSYAAFS KEVPGFKL SKSHLLRI LFCSFVFM FLVVFLKK ILILNMYF EAALIMFV SFTVYFSL GIFVFKIIN IKAIKEMI GEIRK			
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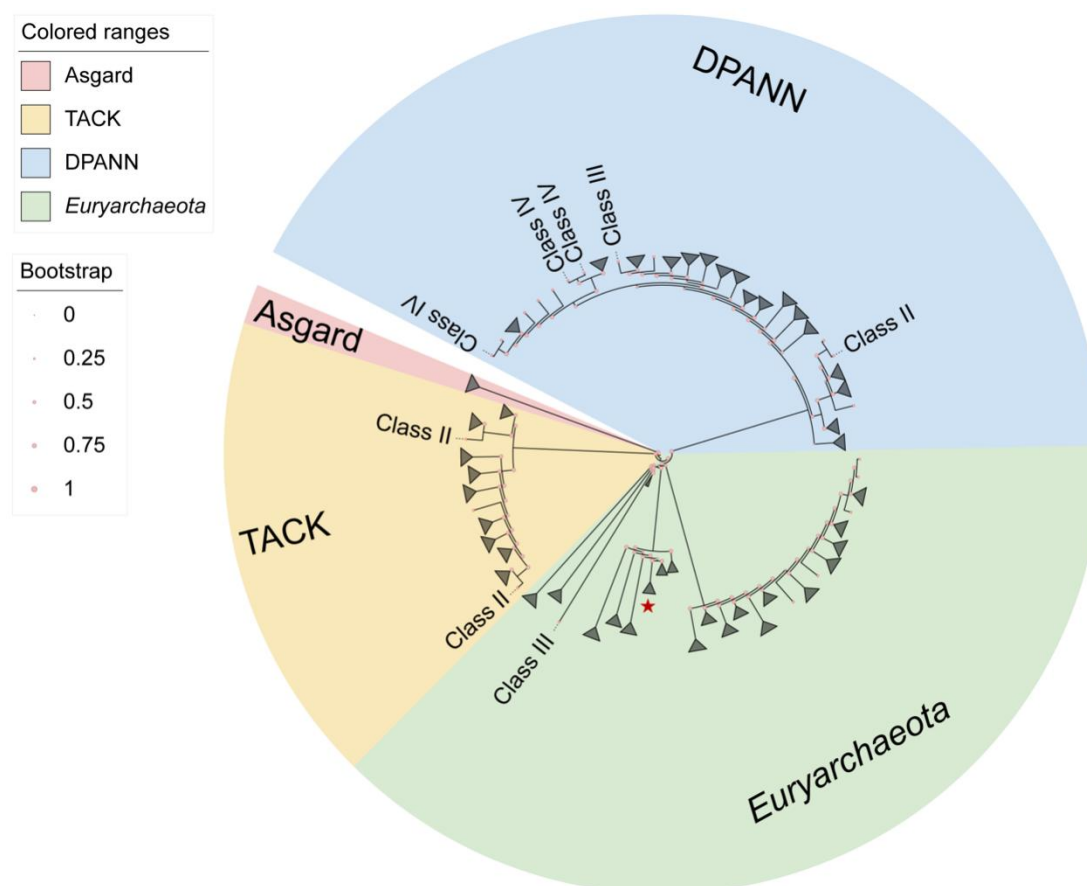


Figure 4-S1. Phylogenetic tree of MatE proteins from 4,351 archaeal genomes. The phylogenetic tree was constructed using all MatE proteins obtained from 4,351 MatE-containing archaeal genomes. For structural classification, a subset of 853 representative MatE proteins was selected, and these strains are classified into four structural classes (Class I–IV). Strains not labeled on the tree are assigned to Class I. Red, yellow, blue, and green indicate the subgroups of Asgard, TACK, DPANN, and Euryarchaeota, respectively. The reference *Methermicoccus shengliensis* MatE is indicated by a red star. Black lines represent phylogenetic branches.

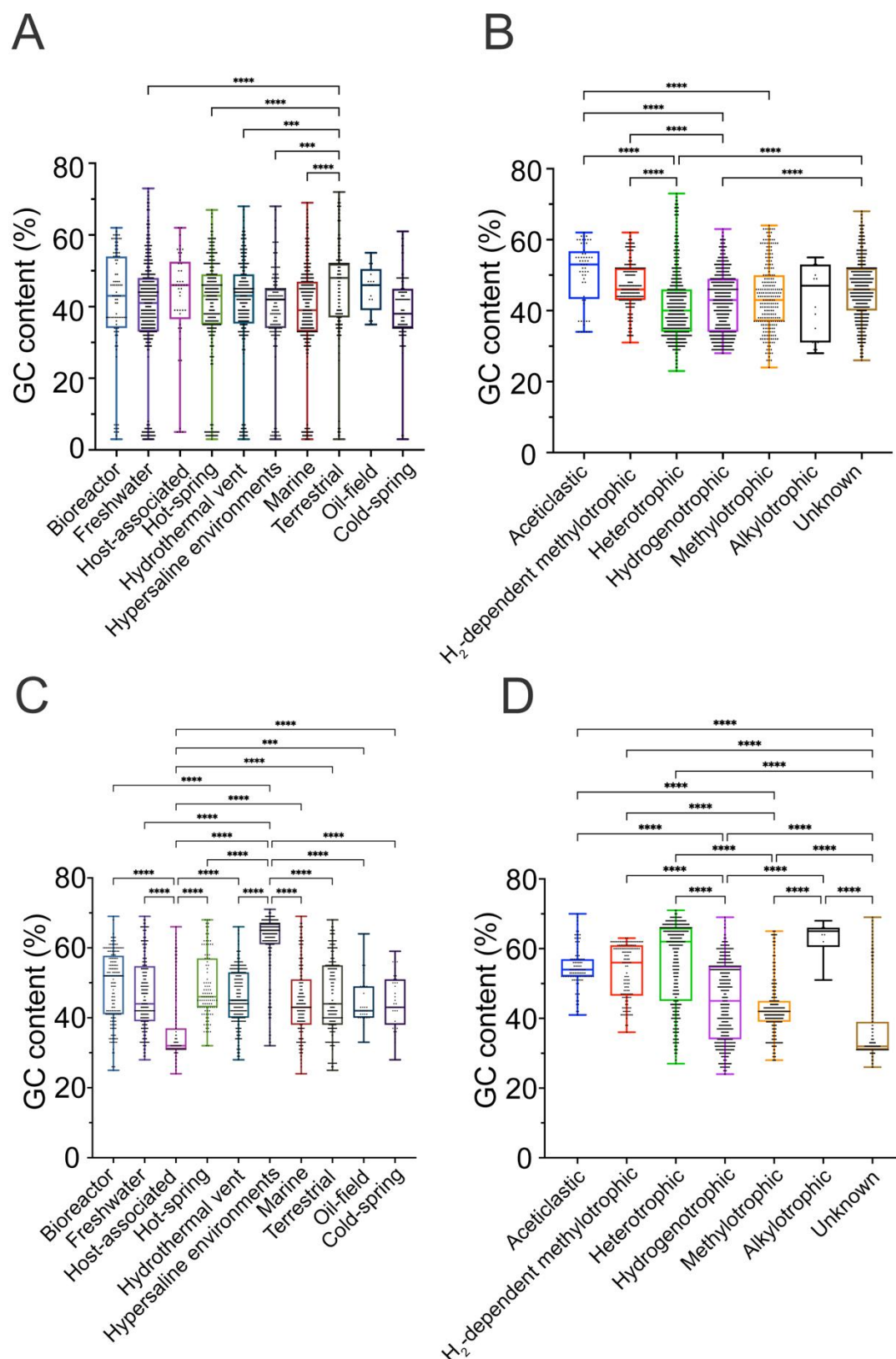


Figure 4-S2. GC content variation across habitats and metabolic types in archaeal genomes with and without MatE. **A.** GC content distributions across habitats in 6,513 archaeal genomes lacking MatE transporters (controls). **B.** GC content distributions

across archaeal metabolic types in the same 6,513 MatE-lacking genomes.

Alkylotrophic and heterotrophic are used as descriptive categories based on genome annotations or literature evidence, whereas H₂-dependent methylotrophic, acetoclastic, hydrogenotrophic, and methylotrophic metabolisms represent canonical archaeal metabolic pathways. Genomes with unassigned metabolic features are classified as Unknown. **C.** GC content distributions across habitats in 4,351 archaeal genomes containing MatE transporters. **D.** GC content distributions across metabolic types in the same 4,351 MatE-containing genomes. Panels **A** and **B** serve as control groups, whereas panels **C** and **D** represent experimental groups. Colors indicate different habitat categories or metabolic types as labeled on the x-axis. In each boxplot, the median is shown in the box as a thick bar (unless it coincides with the borderline). The 25th and 75th percentiles are, respectively, in the lower and upper bounds of the box. Lines through the boxes indicate the minimum value and maximum values, while the whiskers correspond to the 1.5 interquartile range from the bounds. Asterisks indicate levels of statistical significance determined by one-way ANOVA with Welch's correction (***, $P < 0.001$; ****, $P < 0.0001$).

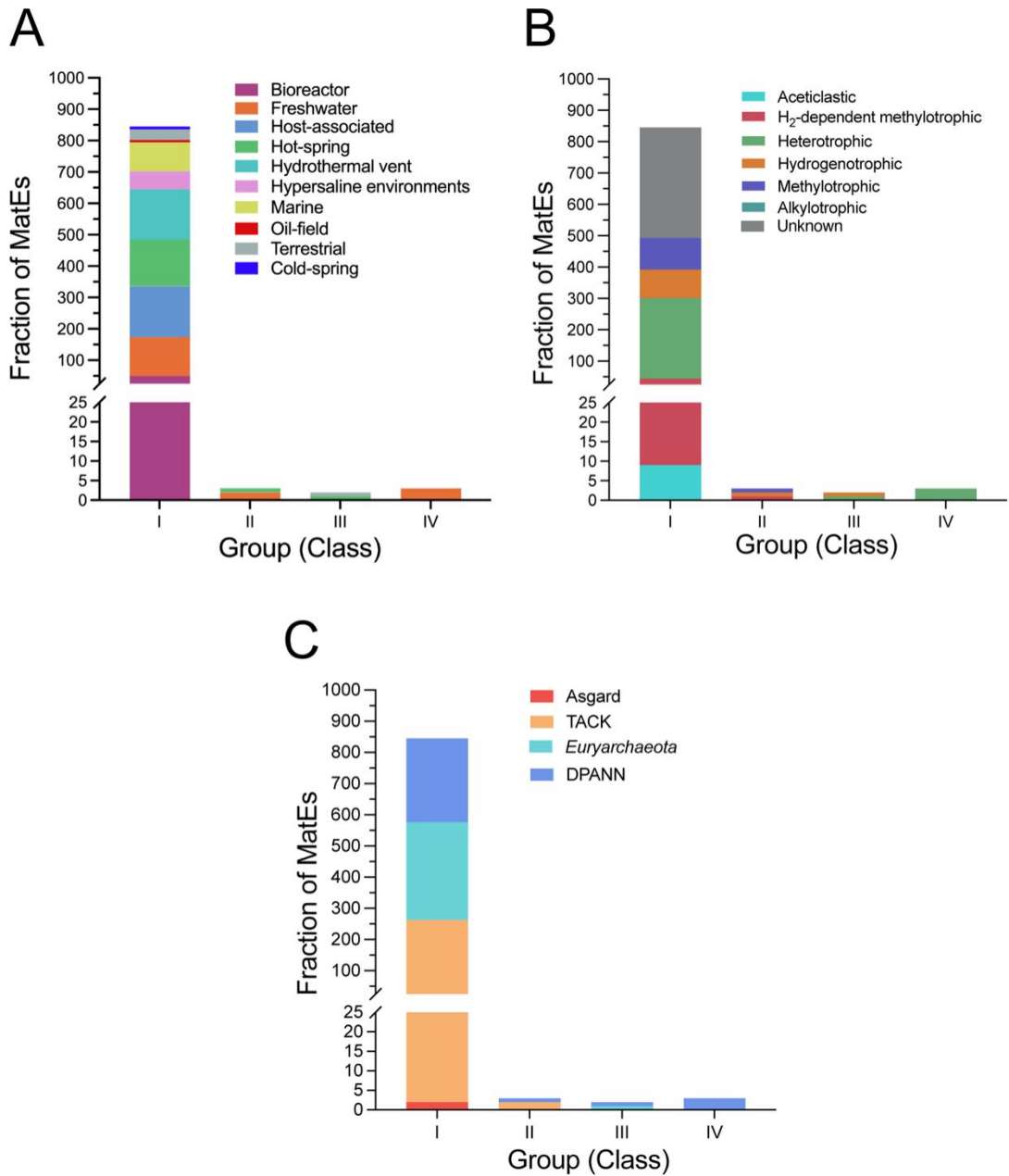


Figure 4-S3. MatEs are involved in habitat adaptation. **A.** The distribution of the four MatE structural classes (Class I–Class IV) across ten different ecological niches in the bar chart. **B.** The distribution of the four MatE structural classes across six metabolic types in the bar chart. **C.** The distribution of the four MatE structural classes (Class I–Class IV) across the four archaeal superphyla in the bar chart.

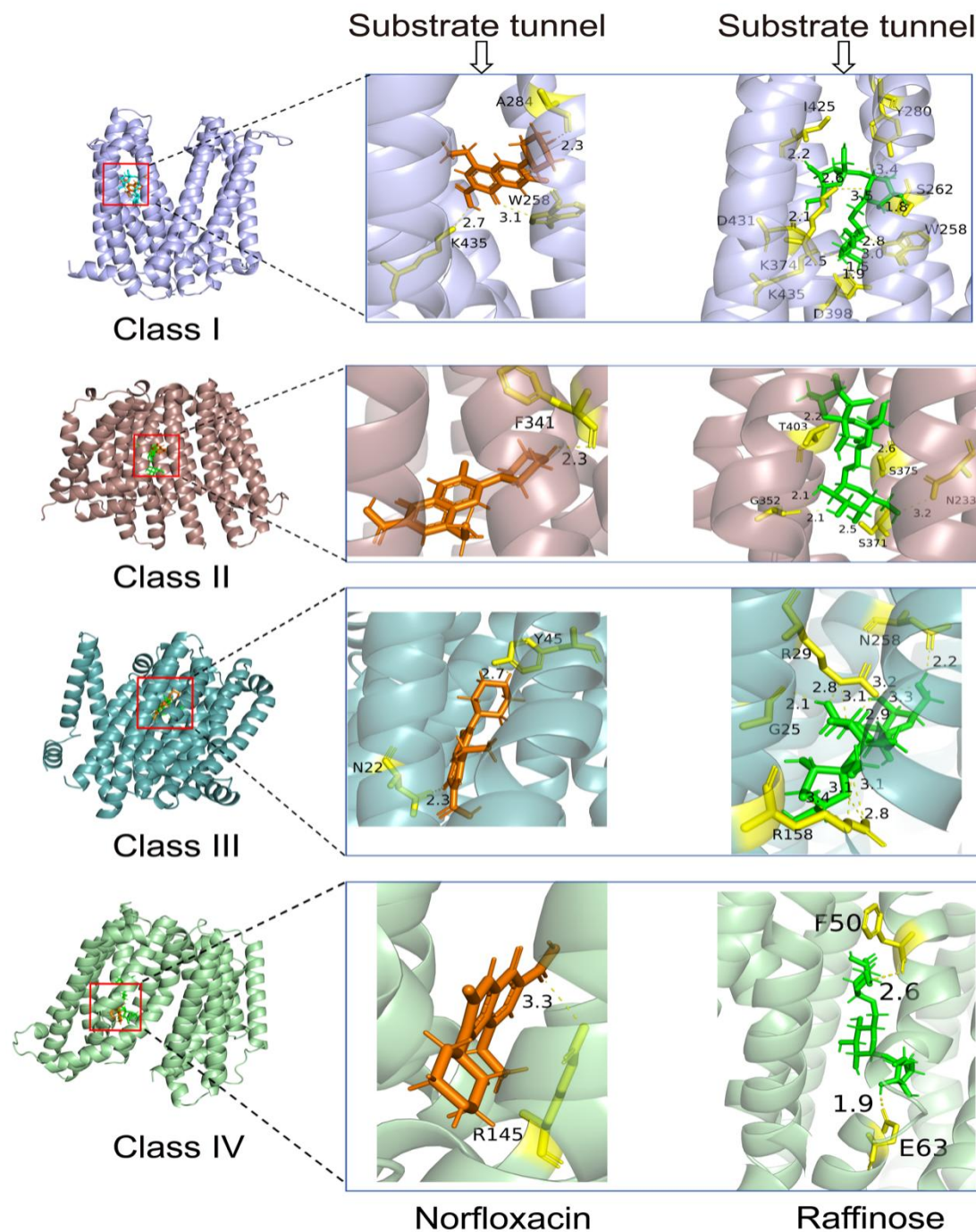


Figure 4-S4. Molecular docking of different classes of MatE transporters with norfloxacin and raffinose. Protein backbones are shown in cartoon representation and colored differently for each class. Substrates are shown as stick models and colored orange (Norfloxacin) and blue (Raffinose). Key interacting residues are shown as yellow sticks. Yellow dashed lines indicate hydrogen bonds, and the numbers adjacent to the dashed lines represent the distances between interacting atoms or residues in the protein, measured in ångströms (Å).

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Chapter 5

**General discussion,
conclusions, and perspectives**

Short overview of chapter V

Chapter V integrates the major findings of Chapters III and IV and discusses their broader biological significance. The general discussion considers the role of MatE in substrate uptake and methanogenesis, its functional relationship with bacterial MatE transporters and other archaeal transporter families, and the evolutionary and ecological significance of archaeal MatE diversity. The chapter concludes by summarizing the main findings and outlining future directions for genetic tool development, functional and ecological characterization, and potential biotechnological applications.

5.1 General discussion

This dissertation investigated archaeal MATE family transporters from functional, structural, and evolutionary perspectives, with particular emphasis on their roles in substrate uptake, methanogenic metabolism, and ecological adaptation. By combining molecular genetics, protein-protein interaction assays, structural modeling, molecular docking, and comparative genomic analyses, this work provides insights into the functional role of MatE in *Methermicoccus shengliensis* and the diversity of MatE transporters across the archaeal domain.

5.1.1 Functional role of archaeal MatE in substrate uptake and methanogenesis

Methanogenic archaea play essential roles in anaerobic carbon cycling and methane production. Although the biochemical pathways of methanogenesis have been extensively investigated, the mechanisms responsible for substrate uptake remain comparatively poorly understood, particularly in methoxydotrophic methanogens that utilize structurally complex methoxylated aromatic compounds.

The findings of this dissertation support a role for MatE in the uptake of methoxylated aromatic compounds in *M. shengliensis*. The impaired growth and methane production observed in the $\Delta matE$ mutant when 2-methoxybenzoate was used as the substrate indicate that efficient substrate transport can directly influence the availability of substrates for methanogenic metabolism. This provides a functional link between membrane transport and methane production and highlights substrate uptake as an important component of methanogenic physiology.

The proposed Tlp-MatE relationship further suggests a possible connection between substrate sensing and membrane transport. The interaction between these proteins is consistent with a model in which Tlp may participate in the recognition of extracellular aromatic substrates and communicate with MatE to facilitate their uptake. Such a regulatory organization could be particularly advantageous for methanogens that encounter structurally diverse aromatic compounds in anaerobic environments. However, the current evidence does not fully resolve the molecular transport mechanism or the complete physiological role of the Tlp-MatE system. Other transporters may also contribute to aromatic substrate uptake, and the relative contribution of individual transport systems remains to be determined.

More broadly, these findings suggest that membrane transport should be considered an integral component of methanogenic metabolism rather than merely a

process supporting nutrient acquisition. For methanogens utilizing complex substrates, transporter activity determines which compounds become accessible to intracellular metabolic pathways and may therefore influence substrate utilization, growth, and methane production.

5.1.2 Functional relationship between archaeal MatE and bacterial MATE transporters

An important question arising from this study is whether archaeal MatE transporters perform functions similarly to those characterized in bacteria.

In bacterial, MATE transporters have been predominantly characterized as multidrug efflux pumps, particularly in the context of antibiotic resistance. These transporters typically export structurally diverse cationic substrates, including ethidium bromide, fluoroquinolones, aminoglycosides, and various dyes, thereby preventing toxic intracellular accumulation and reducing bacterial susceptibility to antimicrobial agents (Garcia Í et al., 2022; He et al., 2010; Miyauchi et al., 2017; Yamamoto et al., 2022). For instance, YoeA, a Na⁺-driven efflux transporter, was identified as a MATE family member and shown to be specifically responsible for the extrusion of plipastatin (Wang et al., 2024). Similarly, AbeM, an H⁺-coupled efflux pump, significantly increases the minimum inhibitory concentrations (MICs) of multiple antibiotics, including ciprofloxacin, norfloxacin, ofloxacin, gentamicin, triclosan, daunorubicin, doxorubicin, ethidium bromide, and rhodamine (Su et al., 2005). Collectively, bacterial MATE transporters play crucial roles in multidrug resistance and the maintenance of cellular homeostasis.

In contrast, experimental evidence supporting efflux-related functions of archaeal MATE transporters remains extremely limited. Although an active antibiotic efflux system has been reported in the halophilic archaeon *Halorubrum amylolyticum* CSM52 (Fakhoury et al., 2024), the specific contribution of its MATE transporters to antibiotic extrusion remains speculative and lacks genetic validation.

The findings of this dissertation therefore provide a different functional perspective on the MATE family. In *M. shengliensis*, MatE is associated with the uptake of methoxylated aromatic compounds rather than the canonical drug-efflux function commonly described in bacteria. This difference suggests that the MatE transporter family has undergone functional diversification across evolutionary lineages and that archaeal MatE proteins may have acquired specialized functions associated with archaeal metabolic strategies, including methanogenesis.

5.1.3 MatE and other transporter families involved in aromatic compound transport

The identification of MatE as a transporter associated with methoxylated aromatic compound uptake also raises the question of whether other transporter families perform similar functions in archaea and bacteria.

The Major Facilitator Superfamily (MFS) are widespread in bacteria and archaea, it is one of the largest known membrane transport families with many members, which can promote the transmembrane transport of solutes such as sugars, drug molecules, peptides, Na⁺ and H⁺ under electrochemical gradient. Although no specific transporters of aromatic compounds have been found in archaea, but the aromatic acid/H⁺ symporter family within MFS has been extensively characterized in bacteria, these includes PcaK from *Pseudomonas putida* for the uptake of protocatechuate and 4-hydroxybenzoate (Nichols et al., 1997; Saier et al., 1999), TfdK from *Ralstonia eutropha* JMP134 (Leveau et al., 1998) for 2,4-dichlorophenoxy acetate uptake, BenK, VanK, PcaK, and MucK from *Acinetobacter baylyi* ADP1 for benzoate uptake (Collier et al., 1997; D'Argenio et al., 1999; Williams et al., 1997), MhbT of *Klebsiella pneumoniae* M5a1 for 3-hydroxybenzoate uptake (Xu et al., 2012) and MhpT from *Escherichia coli* K-12 for 3-hydroxyphenyl uptake (Xu et al., 2013).

Unlike MFS transporters, ABC transporters utilize the energy derived from ATP binding and hydrolysis to perform efflux functions, and they are found in almost all organisms. These transporters function in both import and export processes. Import-type ABC transporters mediate the uptake of essential nutrients such as peptides, vitamins, amino acids, transition metals, and osmoprotectants to enhance virulence. Export-type ABC transporters promote virulence by exporting essential molecules involved in the synthesis of sugar complexes (e.g., lipopolysaccharides and capsular polysaccharides) as well as exogenous toxic compounds. In bacteria, several studies have implicated ABC transport systems in the uptake of aromatic compounds. For example, solute binding proteins (SBPs) from *Rhodopseudomonas palustris* have been shown to recognize lignin-derived aromatic degradation products (Giuliani et al., 2011; Hosaka et al., 2013; Michalska et al., 2012). Genetic evidence further supports the involvement of ABC transporters in *o*-phthalate (OPA) uptake in *Burkholderia* strains and *Rhodococcus jostii* RHA1 (Chang et al., 2009; Hara et al., 2010). Additional studies suggest ABC-dependent transport of homogentisate in *Pseudomonas putida* U (Arias-Barrau et al., 2005), 2-chlorobenzoate in *Pseudomonas huttiensis* D1 (Yuroff et al., 2003), 4-hydroxybenzoate in *Acinetobacter* sp. BEM2

(Allende et al., 2000), and 4-hydroxyphenylacetate in *Klebsiella pneumoniae* M5a1 (Allende et al., 1992).

In archaea, although MFS and ABC transporters are widely distributed, specific members experimentally demonstrated to mediate aromatic compound uptake have not yet been established. In contrast, the present study identified the MatE transporter from *M. shengliensis* ZC-1 as a component associated with the uptake of methoxylated aromatic compounds (Leng et al., 2025). This finding expands the known functional repertoire of the MATE family beyond its well-established efflux functions in bacteria and suggests that archaea may employ distinct transporter systems for aromatic substrate acquisition.

Nevertheless, the involvement of other transporter families cannot be excluded. Previous studies have identified MFS transporters located near the Mto proteins in *M. shengliensis* and proposed that these proteins may contribute to the transport of methoxylated aromatic compounds (Kurth et al., 2020). Therefore, future experimental characterization of these candidate transporters will be important for determining whether multiple transporter systems cooperate in aromatic substrate uptake.

5.1.4 Evolutionary diversity, structural conservation, and ecological distribution of archaeal MatE

The functional characterization of MatE in *M. shengliensis* provides a specific example of archaeal MatE function, while the comparative analyses presented in this dissertation place this finding within a broader evolutionary context. The uneven distribution of MatE across archaeal lineages, habitats, and metabolic lifestyles suggests that its occurrence is not simply a consequence of broad taxonomic inheritance. Instead, the retention and diversification of MatE may be influenced by the genomic and physiological characteristics of individual archaeal lineages.

The association of MatE with larger genomes may reflect a relationship between transporter repertoire and overall genomic or metabolic complexity. Organisms with larger genomes often possess broader metabolic capacities and more extensive regulatory and transport systems, potentially allowing them to exploit a wider range of environmental resources. In this context, MatE may represent one component of a broader transport capacity associated with metabolic versatility rather than a transporter restricted to a particular habitat.

The enrichment of MatE among host-associated and hypersaline archaea further suggests that environmental conditions may influence the retention or diversification of these transporters. Host-associated environments can contain chemically diverse organic compounds, while hypersaline environments impose strong physicochemical constraints on cellular physiology. The presence of MatE across such contrasting environments suggests that its functional importance may extend beyond a single ecological niche. However, the observed associations do not by themselves establish direct causal relationships between MatE and specific environmental conditions, and further ecological and physiological studies will be required to determine the selective forces underlying these distribution patterns.

The structural analysis also reveals an interesting balance between conservation and diversification. The strong predominance of Class I indicates that one structural architecture has been maintained across a broad range of archaeal lineages, suggesting that this configuration may provide a conserved and broadly compatible framework for MatE transporter function. In contrast, the less common Classes II-IV may represent more specialized evolutionary trajectories. Their distinct structural and functional associations raise the possibility that some archaeal MatE homologs have diverged from the dominant MatE architecture to perform specialized transport-related functions.

The predicted differences in ligand-interacting residues among the structural classes further suggest that structural diversification may contribute to differences in substrate recognition. However, molecular docking provides only a prediction of potential ligand interactions and cannot establish transport activity, substrate specificity, or transport direction. Experimental transport assays and targeted mutagenesis will therefore be necessary to determine whether the observed structural differences correspond to distinct physiological functions.

Taken together, the findings from the functional and comparative analyses suggest that archaeal MatE transporters can be considered at two complementary levels. At the cellular level, MatE in *M. shengliensis* is associated with the uptake of methoxylated aromatic compounds and with methane production. At the evolutionary level, archaeal MatE proteins display both strong structural conservation and functional diversification across different lineages and environmental contexts. These observations support the view that membrane transport systems contribute not only to substrate acquisition but also to the metabolic flexibility and ecological adaptation of archaea.

5.2 Conclusion

This dissertation investigated archaeal MATE family transporters from functional, structural, and evolutionary perspectives, with particular emphasis on their roles in substrate uptake and methane metabolism.

In *M. shengliensis*, MatE is associated with the uptake of methoxylated aromatic compounds and contributes to growth and methane production when 2-methoxybenzoate is used as a substrate. These findings provide evidence for an important role of membrane transport in substrate utilization and methanogenic metabolism.

At the archaeal domain level, MatE transporters show broad but uneven distribution across archaeal lineages, habitats, and metabolic types. Their distribution and structural diversity are associated with differences in genomic characteristics and environmental contexts, with a highly conserved dominant structural alongside less common structural class.

Overall, this dissertation establishes MatE as a functionally relevant archaeal transporter system and highlights the importance of membrane transport in archaeal metabolic flexibility and ecological adaptation. It provides a basis for further investigation of the functional diversity, transport mechanisms, and ecological roles of archaeal MATE transporters.

5.3 Perspectives

This study establishes the MATE system as a previously unrecognized component of substrate metabolism in methanogenic archaea and suggests its broader relevance to environmental adaptation. By linking transporter-mediated substrate uptake to methane production and ecological distribution, this work extends current understanding of archaeal membrane transport systems and provides a conceptual bridge between molecular mechanism and ecosystem-level function.

While these findings lay both theoretical and experimental foundations for elucidating the role of MatE transporters in archaea, they also expose important knowledge gaps. Addressing these gaps will be essential for advancing mechanistic insight, evolutionary interpretation, and potential biotechnological exploitation of archaeal transport systems.

5.3.1 Genetic manipulation techniques in archaea

Genetic manipulation in archaea, particularly in methanogens, remains less developed and less versatile than in bacterial systems. Early genetic tools were established in model methanogens, including natural transformation (Bertani et al., 1987) and electroporation in *Methanococcus voltae* (Micheletti et al., 1991), followed by protoplast-based transformation (Patel et al., 1993; Patel et al., 1994). PEG-mediated transformation was subsequently developed for *Methanococcus maripaludis* (Tumbula et al., 1994), while liposome-mediated DNA delivery provided an alternative approach for *Methanosarcina acetivorans* and *M. voltae* (Metcalf et al., 1997; Thomas et al., 2001). These developments established the foundation for genetic manipulation in methanogens, but transformation efficiency and the availability of versatile genetic tools remain important limitations.

In the present study, both PEG-mediated and liposome-mediated transformation were evaluated for *M. shengliensis*, and gene knockout was successfully achieved using liposome-mediated transformation. However, the low number of positive monoclonal mutants indicates that genetic manipulation in this thermophilic methanogen remains technically challenging. Improving transformation efficiency and developing reliable gene complementation systems will therefore be important for further functional characterization of MatE and other candidate transporters.

Future research should focus on developing more efficient and versatile genome-editing tools adapted to the anaerobic and thermophilic physiology of methanogens. CRISPR-based genome editing, inducible expression systems, and promoter engineering could provide valuable approaches for systematic functional characterization. Such tools would facilitate the validation of transporter functions, investigation of regulatory networks, and analysis of the coordination between substrate transport, methanogenesis, and cellular energy metabolism.

5.3.2 Functional diversity

This study provides evidence that MatE contributes to the uptake of methoxylated aromatic compounds and methane production in *M. shengliensis*. However, the functional diversity of the broader archaeal MatE family remains largely unexplored. Comparative genomic and structural analyses suggest that different MatE homologs may be associated with diverse transport functions, including the transport of multidrug/oligosaccharidyl-lipid/polysaccharide (MOP)-related compounds, organic acids, or metal ions. The distinct structural classes

identified in Chapter 4 may therefore reflect functional diversification among archaeal MatE transporters.

Future studies combining heterologous expression, direct transport assays, high-throughput substrate screening, and metabolomics will be important for defining the substrate spectrum and physiological functions of different MatE homologs. Particularly, experimental characterization of representative proteins from different structural classes could determine whether their structural differences correspond to distinct substrate specificities and transport mechanisms.

5.3.3 Ecological diversity

The broad distribution of MatE transporters across archaeal lineages, including their enrichment in host-associated and hypersaline environments, suggests that these transporters may contribute to archaeal ecological adaptation. However, their specific ecological functions under natural conditions remain largely unresolved.

Future studies combining metagenomics, single-cell omics, and in situ transcriptomics could help characterize MatE systems in uncultured archaea and clarify their roles in natural environments. Investigating the environmental factors regulating *matE* expression may further reveal how archaeal transporter systems contribute to adaptation to complex ecological conditions and participate in broader biogeochemical cycles.

5.3.4 Biotechnological applications

The involvement of MatE in methoxylated aromatic compound uptake suggests potential opportunities for biotechnological applications. In synthetic biology, MatE-mediated transport systems could be explored to improve substrate uptake in engineered methanogens or other microbial hosts, potentially facilitating the conversion of lignin-derived or other aromatic substrates into methane or other valuable products.

The potential functional diversity and stress-related roles of archaeal MatE transporters may also provide opportunities for developing robust microbial cell factories adapted to challenging industrial conditions. Future studies could investigate the functional plasticity of MatE in engineered microbial chassis and assess whether transporter engineering can be used to modulate substrate availability and metabolic flux.

Artificial intelligence and machine learning approaches could further complement experimental studies by predicting transporter-substrate interactions, identifying structural determinants of transporter function, and guiding the design of engineered transport systems. Combining experimental characterization with structure-based and data-driven approaches may accelerate the discovery and functional characterization of MatE transporters, including those from uncultured archaea.

Overall, further characterization of archaeal MatE transporters may contribute to both a better understanding of archaeal membrane transport and the development of microbial platforms for sustainable biotechnological applications.

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