

Genome analysis of  
*Zymomonas mobilis* and *Gracilibacillus spp.*

# INTRODUCTION

*Zymomonas* species are **Gram-negative**, **non-endospore-forming**, catalase-positive, **aerotolerant**, facultative anaerobic bacteria. Morphologically, these bacteria are short plump rods that occur singly, in pairs, and sometimes in chains or rosettes.

*Zymomonas* is **ethanol tolerant** (below 10% ethanol v/v) and grows optimally at **pH above 3.4** and temperature of 25–30 °C (van Vuuren, Cosser, & Prior, 1980). These bacteria **metabolise glucose and fructose as a source of carbon but are unable to utilise maltose and maltotriose**.

*Zymomonas* species are often isolated as a **source of spoilage microorganisms** from various traditional alcoholic beverages throughout the world. These bacteria are found on the **glucose-rich sap of agave, sugar cane and palm trees** as a naturally occurring fauna. *Zymomonas* is a biotechnologically important microorganism for **industrial production of fuel ethanol**.

## Taxonomical significance:

*Zymomonas* is a genus under the phylum Proteobacteria, class Alphaproteobacteria, order Sphingomonadales, and family Sphingomonadaceae. It contains a single species, *Z. mobilis*, formerly known as *Achromobacter anaerobium*, first isolated from beer. Historical synonyms include *Saccharomonas lindneri* and *Pseudomonas lindneri*. Currently, three subspecies are recognized—*Z. mobilis* subsp. *mobilis*, *pomaceae*, and *francensis*—distinguished by phenotypic, protein, and genetic traits, as well as growth at 36 °C. Among them, only *Z. mobilis* subsp. *mobilis* is associated with beer spoilage.

## Metabolic aspects:

Experimental studies have shown that *Zymomonas mobilis* (ZM4) lacks a functional Embden–Meyerhof–Parnas (EMP) pathway or glycolytic pathway due to the **absence of the phosphofructokinase gene**, despite possessing genes for other glycolytic enzymes. Instead, *Z. mobilis* utilizes the Entner–Doudoroff (ED) pathway **anaerobically to ferment** glucose, fructose, and sucrose into **ethanol and CO<sub>2</sub>**. It cannot metabolize lactose, maltose, or cellobiose owing to missing metabolic genes. Genome analysis also indicates the absence of 2-oxoglutarate dehydrogenase and malate dehydrogenase from the TCA cycle, suggesting the presence of an alternative pathway for synthesizing intermediates such as oxaloacetate, malate, and succinate.

<i>Zymomonas mobilis</i>	
Scientific classification	
Domain:	Bacteria
Kingdom:	Pseudomonadati
Phylum:	Pseudomonadota
Class:	Alphaproteobacteria
Order:	Sphingomonadales
Family:	Zymomonadaceae Hördt et al. 2020 <sup>[1]</sup>
Genus:	<i>Zymomonas</i> Kluyver and van Niel 1936 (Approved Lists 1980)
Species:	<i>Z. mobilis</i>

# INTRODUCTION

*Gracilibacillus* species are **gram-positive**, **endspore forming**, **rod shaped**, **aerobic bacteria** that is **motile** by use of a flagellum and exhibits catalase, but not oxidase activity.

## Habitat:

*Gracilibacillus* species are moderately halophilic to halotolerant bacteria — meaning they require or tolerate significant salt concentrations for optimal growth. Their natural environments are typically saline or hypersaline ecosystems where osmotic pressure is high and nutrients are limited. Some strains have been reported from marine sediment samples and coastal seawater, consistent with the genus's halotolerant physiology.

## Taxonomy:

*Gracilibacillus* is a genus under the phylum Bacillota, class Bacilli, order Bacillales, family amphibacillaceae.

The history of the genus *Gracilibacillus* is relatively recent in bacterial taxonomy but reflects major developments in the classification of Gram-positive, spore-forming, halophilic bacteria. The genus *Gracilibacillus* was first proposed in 1996 by Wainø, Tindall, and Ingvorsen. It was separated from the genus *Bacillus* because of distinct physiological and genetic characteristics, especially moderate halophilicity and unique 16S rRNA gene sequences.

## Function:

*Gracilibacillus* species play a role in nutrient cycling by decomposing organic matter and aiding in the recycling of carbon, nitrogen, and phosphorus. Being aerobic bacteria, they require oxygen for growth and help break down complex compounds in oxygen-rich environments, contributing to soil and marine ecosystem balance.

<i>Gracilibacillus</i>	
Scientific classification	
Domain:	Bacteria
Kingdom:	Bacillati
Phylum:	Bacillota
Class:	Bacilli
Order:	Bacillales
Family:	Amphibacillaceae
Genus:	<i>Gracilibacillus</i>

Wainø et al. 1999

Zymomonas mobilis subsp. mobilis str. CP4 = NRRL B-14023	USA
Zymomonas mobilis strain ZM401	South Korea
Zymomonas mobilis subsp. mobilis ZM4 = ATCC 31821	China
Zymomonas mobilis subsp. pomaceae NBRC 13757	United Kingdom
Gracilibacillus salitolerans strain SCU50	China
Gracilibacillus caseinilyticus strain SSWR10-1	Thailand
Gracilibacillus SALINARUM strain SSPM10-3	South Korea
Gracilibacillus sp. Marseille-P2481 strain Marseille-P2481T	France

# RATIONALE OF WORK

This study leverages whole-genome sequencing and advanced bioinformatics to investigate the genomic basis of antimicrobial resistance (AMR) and metabolic potential in four strains each of the industrially relevant *Zymomonas mobilis* and 4 strains of the environmentally resilient *Gracilibacillus*. While *Z. mobilis* is critical for biofuel production, its AMR profile is poorly understood. *Gracilibacillus*, thriving in extreme niches, may harbor novel resistance mechanisms. Using PROKKA for annotation and the CARD database, we will systematically identify AMR genes, classify them by drug mechanism (e.g., efflux, inactivation) and gene family, and critically analyze hypothetical proteins to propose novel resistance mechanisms.

## Rationale for Organism Selection:

- ***Zymomonas mobilis*:** This bacterium is a well-known ethanologen with a high metabolic rate for sugar fermentation, making it a prime candidate for biofuel production. However, its industrial deployment is often hampered by susceptibility to microbial contaminants and inhibitors present in lignocellulosic hydrolysates. A comprehensive genomic analysis of multiple *Z. mobilis* strains is crucial to:
  - Identify intrinsic and potential acquired AMR genes that could inform strategies for designing robust industrial strains or controlling their spread in bioprocessing environments.
  - Elucidate its full metabolic potential beyond ethanol production, which may reveal novel pathways for bioconversion.
- ***Gracilibacillus*:** Species within this genus are often halotolerant or halophilic, thriving in high-stress environments like salt lakes, saline soils, and fermented foods. These extreme niches are hotspots for the evolution of novel survival mechanisms, including antibiotic production and resistance. Studying *Gracilibacillus* is justified because:
  - Their resilience in harsh conditions suggests the presence of unique resistance mechanisms that are poorly documented.
  - They represent an untapped reservoir of novel genes that could contribute to the understanding of AMR evolution in non-clinical environments, a critical aspect of the One Health approach to combating AMR.

# OBJECTIVE

- The genome analysis focuses on understanding bacterial genome organization and resistance potential through detailed genome annotation and comparative analysis between two different bacterial species.
- The annotated bacterial genome was analyzed using PROKSEE along with CARD (Comprehensive Antibiotic Resistance Database) and Prokka to identify antimicrobial resistance gene families, resistance mechanisms, and protein-coding sequences.
- The analysis aims to establish relationships between genome size, number of annotations, and hypothetical proteins, providing insights into gene density and functional diversity.
- Findings from genome annotation are correlated with ecological adaptations, evolutionary relationships, and functional modules to understand the role of co-adapted genes in bacterial physiology. This approach enables a deeper understanding of the genetic basis of antibiotic resistance, metabolic versatility, and environmental survival mechanisms in bacteria.
- The overall objective is to integrate genomic data to enhance knowledge of bacterial function, resistance mechanisms, and their potential impact on antibiotic resistance surveillance and gene prediction.

# METHODOLOGY

*Zymomonas mobilis* and *Gracilibacillus* were the 2 organisms assigned to our group (Group 4). The following steps were followed to proceed with the genome analysis.

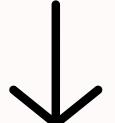
Searched the NCBI website for *Zymomonus mobilis* and *Gracilibacillus spp*



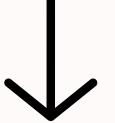
Two different strains of *Zymomonus mobilis* and two different strains of *Gracilibacillus spp* were chosen each having different geographical location



The GenBank Accession Number for each organism was also noted. The GenBank Accession Number for each organism was put on Proksee and the genome map was obtained.



Under Proksee, many analysing tools were used to collect data for the respective species like CARD Resistance Gene Identifier, Prokka, Map Builder and eventually generate a genome map



Necessary information from these tools were stored in corresponding files formats for further analysis. Corresponding data analysis for a tool is discussed later.

# **MATERIALS**

## **CARD Resistance Gene Identifier**

Purpose of the tool: The Comprehensive Antibiotic Resistance Database (CARD) Resistance Gene Identifier (RGI)

URL of the tool: <https://card.mcmaster.ca>

Methodology: The tool was run and output.txt file was downloaded and opened in MS Excel. Two important parameters, Resistance Mechanism & AMR Gene Family was analysed. Common elements from these parameters were found out from the two strains of each organism. Represented as Venn diagrams between the different species as discussed in the results section.

## **Proksee ORFs**

Purpose of the tool: Find Open Reading Frames (ORFs)

URL of the tool: <https://proksee.ca/tools/orfs>

Methodology: PNG files of the genome map with the ORFs labelled were downloaded for all the eight species and are included in the results section for visualisation.

# MATERIALS

## **Prokka**

Purpose of the tool: Annotate the genome sequence with Prokka

URL of the tool: <https://github.com/tseemann/prokka>

Methodology: The tool was run for genome of each of the eight species and prokka.tsv & prokka.gbk files were downloaded. prokka.tsv was opened in MS Excel and the locus tags for only hypothetical proteins were segregated. Then, according to the length of the base pair, ranges (300-1200) were selected for each species. 45 hypothetical proteins were chosen (5 from each range) for a specific species. The sequence of these proteins were retrieved from the prokka.gbk file by opening it with Notepad++ (Version 8.5.8).

45 such hypothetical proteins were annotated using three databases:

- InterPro (<https://www.ebi.ac.uk/interpro/search/sequence>),
- STRING (<https://string-db.org>),
- CELLO (<https://cello.life.nctu.edu.tw/>)

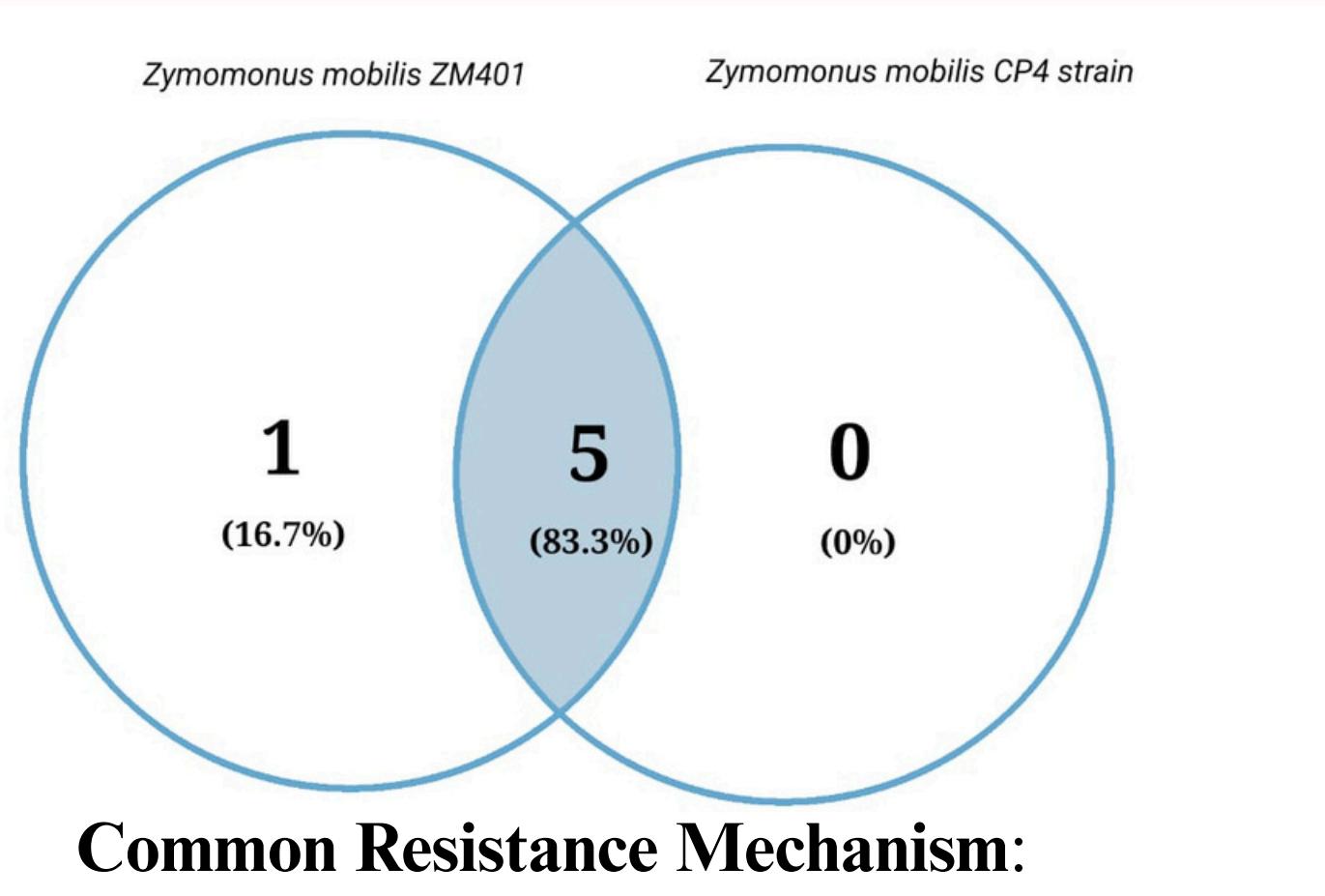
Complete annotation of each protein was noted. Thus, we have annotated 360 proteins (8 species × 45 hypothetical proteins) for the *Zymomonas mobilis* and *Gracilibacillus* spp. Results are represented in the form of pie charts showing different aspects of the annotation in the next section.

# RESULTS

## Antibiotic Resistance Genes (Data analysed from CARD Resistance Gene Identifier)

- Resistance Mechanism:

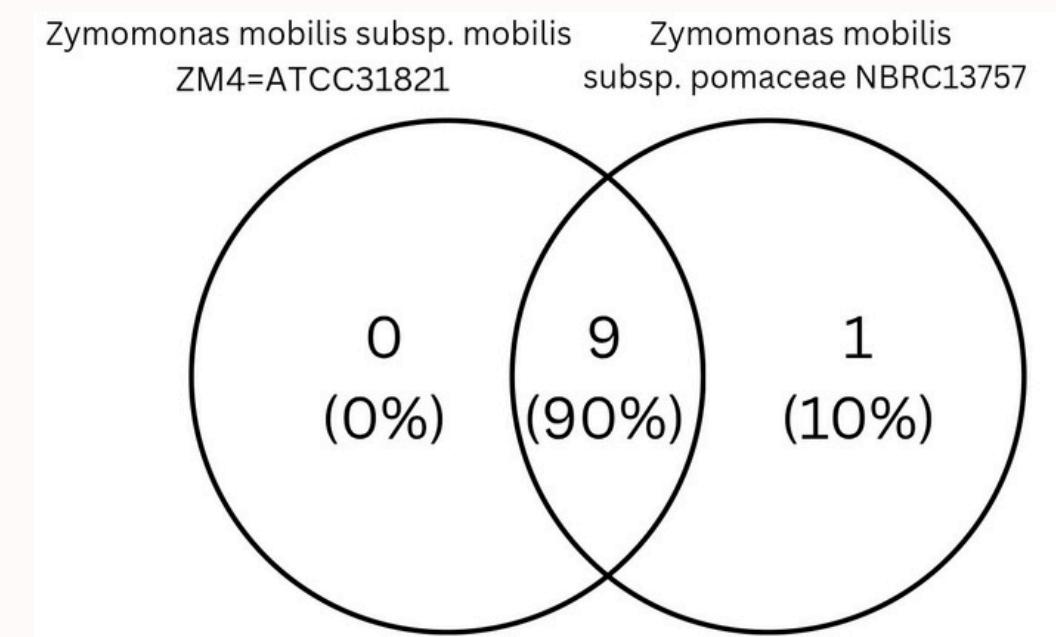
### A. *Zymomonas mobilis* ZM401 strain & *Zymomonas mobilis* CP4 strain



### Common Resistance Mechanism:

- antibiotic efflux
- antibiotic inactivation
- antibiotic target alteration
- antibiotic target replacement
- reduced permeability to antibiotic
- resistance by host-dependent nutrient acquisition

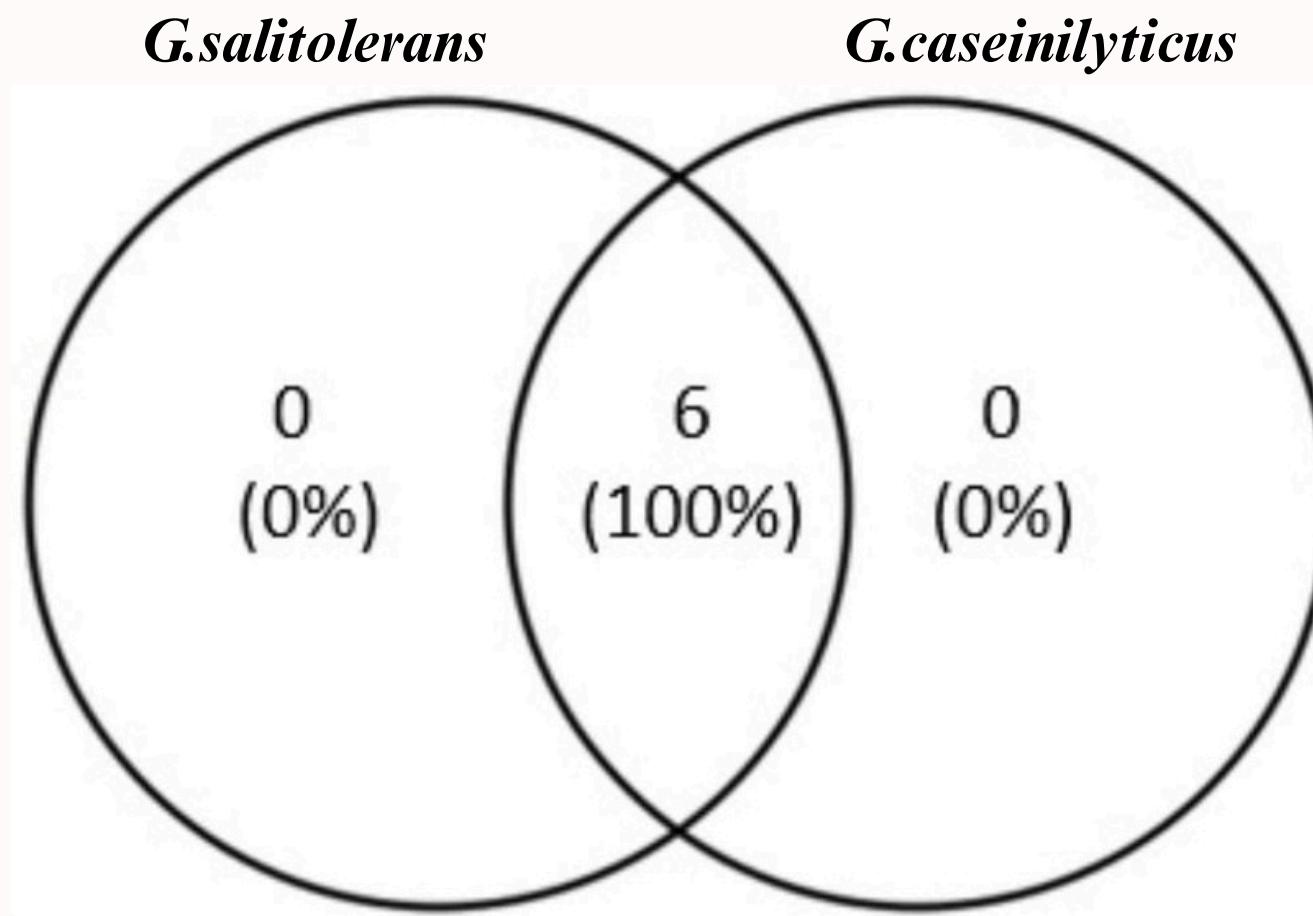
### B. *Zymomonas mobilis* subsp. *mobilis* ZM4 = ATCC 31821 & *Zymomonas mobilis* subsp. *pomaceae*



### Common Resistance Mechanism:

- antibiotic efflux
- antibiotic inactivation
- antibiotic target alteration
- antibiotic target replacement
- antibiotic target protection
- antibiotic efflux; reduced permeability to antibiotic
- antibiotic target alteration, antibiotic efflux
- antibiotic target alteration, antibiotic target replacement
- reduced permeability to antibiotic
- resistance by host-dependent nutrient acquisition

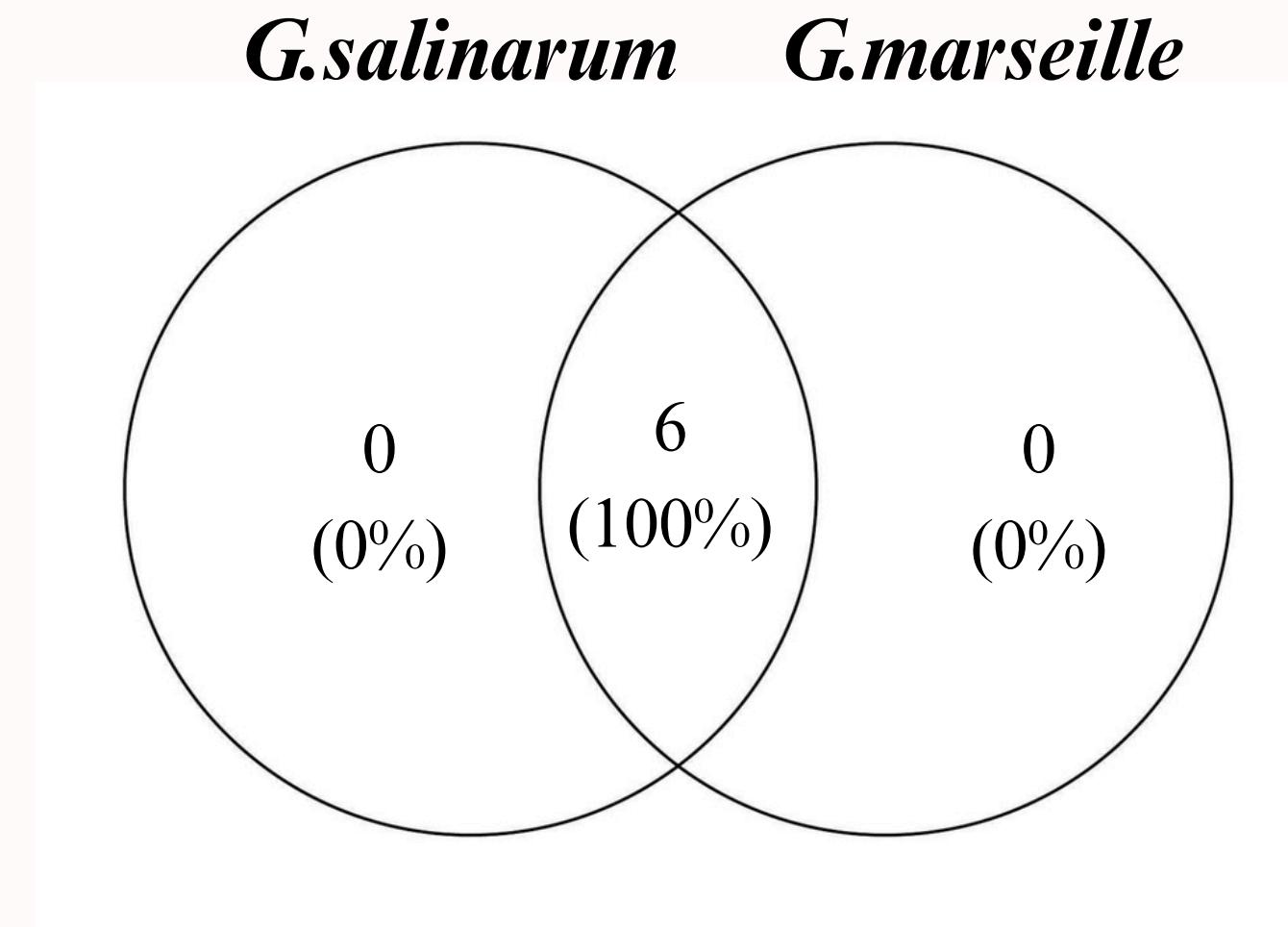
### **C.*Gracilibacillus salitolerans* & *Gracilibacillus caseinilyticus***



#### Common resistance mechanism

1. Antibiotic efflux
2. Antibiotic inactivation
3. Antibiotic target alteration
4. Antibiotic target replacement
5. Antibiotic target protection
6. Reduced permeability to antibiotic

### **D.*Gracilibacillus salinarum* & *Gracilibacillus marseille***



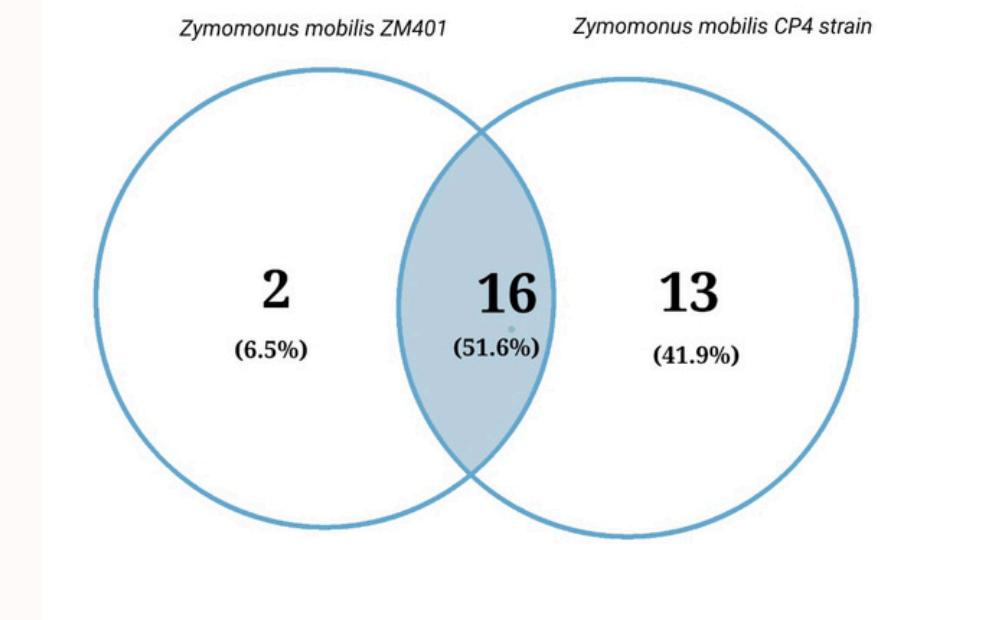
#### Common resistance mechanism

1. Antibiotic efflux
2. Antibiotic inactivation
3. Antibiotic target alteration
4. Antibiotic target protection
5. Antibiotic target replacement
6. Reduced permeability to antibiotic

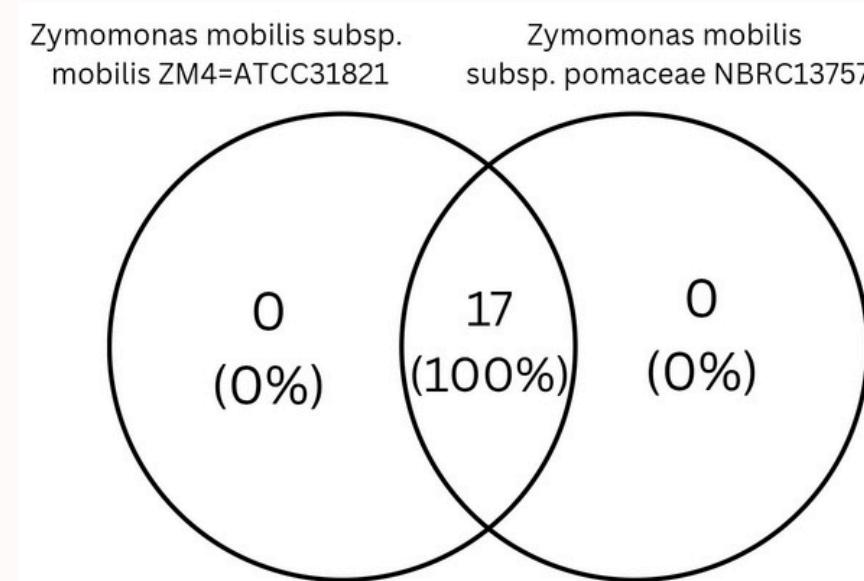
# **RESULTS**

## **Antibiotic Resistance Genes (Data analysed from CARD Resistance Gene Identifier)**

- Antimicrobial Resistance (AMR) gene family
  - A. **Zymomonas mobilis ZM401 strain & Zymomonas mobilis CP4 strain**



- B. ***Zymomonas mobilis* subsp. *mobilis* ZM4 = ATCC 31821 & *Zymomonas mobilis* subsp. *pomaceae***



### **Common AMR gene families:**

1. ATP-binding cassette (ABC) antibiotic efflux pump
2. Erm 23S ribosomal RNA
3. Glycopeptide resistance gene cluster;
4. Major facilitator superfamily (MFS) antibiotic efflux pump
5. Miscellaneous ABC-F subfamily ATP-binding cassette ribosomal protection proteins
6. OXA beta-lactamase
7. Resistance-nodulation-cell division (RND) antibiotic efflux pump
8. Tetracycline inactivation enzyme
9. Tetracycline-resistant ribosomal protection protein
10. Trimethoprim resistant dihydrofolate reductase (dfr)

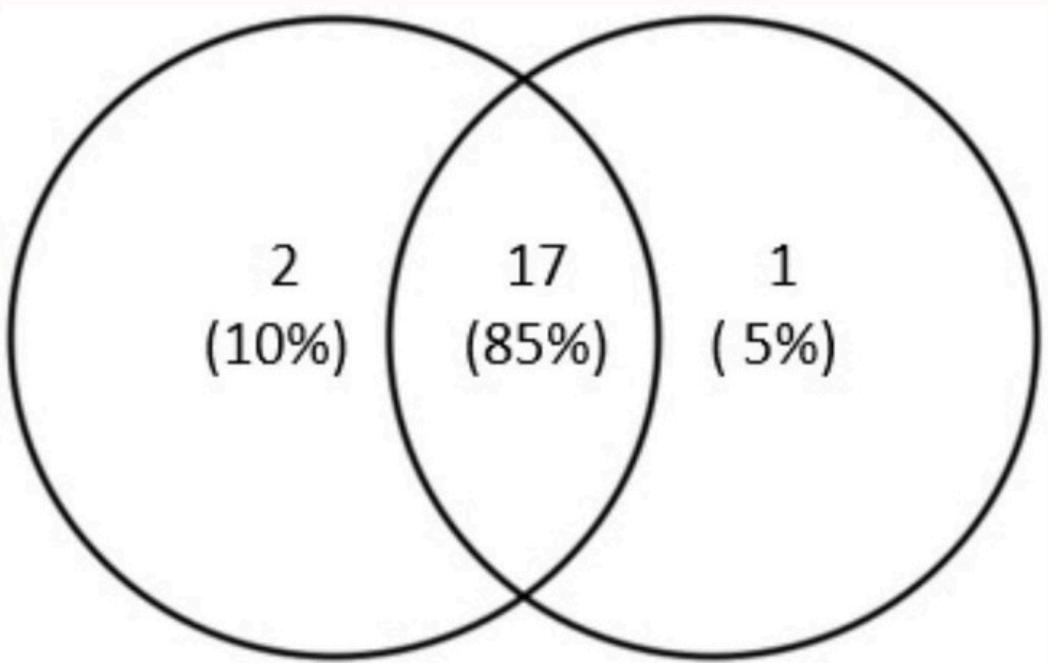
### **Common AMR Gene families**

- Major Facilitator superfamily (MFS) antibiotic efflux pump
- Resistance Nodulation Cell Division(RND) antibiotic efflux pump
- Tetracycline Resistant Ribosomal protection protein
- OXA beta-lactamase
- Miscellaneous ABC-F subfamily ATP-binding cassette ribosomal protection protein
- Glycopeptide Resistance gene cluster
- Trimethoprin Resistant Dihydrofolate reductase dfr
- ADC beta-lactamase pending classification of carbapenemase activity
- Erm 23S ribosomal RNA methyltransferase
- msr-type ABC-F protein
- pmr phosphoethanolamine transferase
- sal-typeABC-F protein
- Rifampin monooxygenase
- Tetracycline Inactivation protein
- Rifamycin-Resistant beta-subunit of RNA polymerase

### **C.*Gracilibacillus salitolerans* & *Gracilibacillus caseinilyticus***

**G.*salitolerans***

**G.*caseinilyticus***



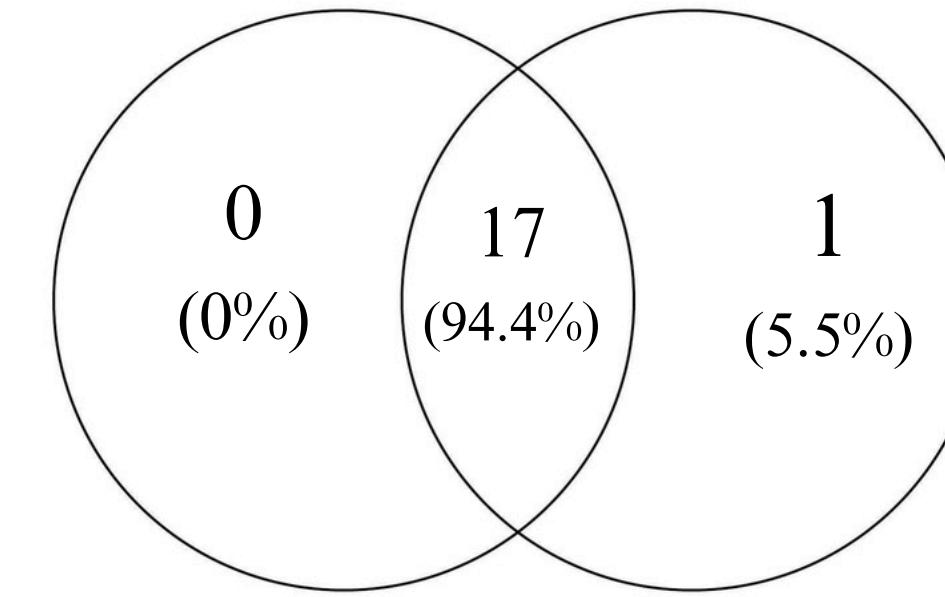
#### Common AMR Gene Family

1. ATP Binding Casette (ABC) antibiotic efflux pump
2. Antibiotic-resistant isoleucyl-tRNA synthetase (ileS)
3. BlaZ beta-lactamase
4. Chloramphenicol acetyltransferase (CAT)
5. Erm 23S ribosomal RNA methyltransferase
6. Glycopeptide resistance gene cluster; vanR
7. Macrolide phosphotransferase (MPH)
8. Major Facilitator Superfamily(MFS) antibiotic efflux pump
9. MCR phosphoethanolamine transferase
10. Miscellaneous ABC-F subfamily ATP-binding cassette ribosomal protection proteins
11. OXA beta- lactamase
12. Resistance-nodulation-cell division (RND) antibiotic efflux pump
13. Rifampin monooxygenase
14. Rifampin phosphotransferase
15. Small multidrug resistance (SMR) antibiotic efflux pump
16. Tetracycline inactivation enzyme
17. Tetracycline-resistant ribosomal protection protein

### **D.*Gracilibacillus salinarum* & *Gracilibacillus marseille***

**G.*salinarum***

**G.*marseille***

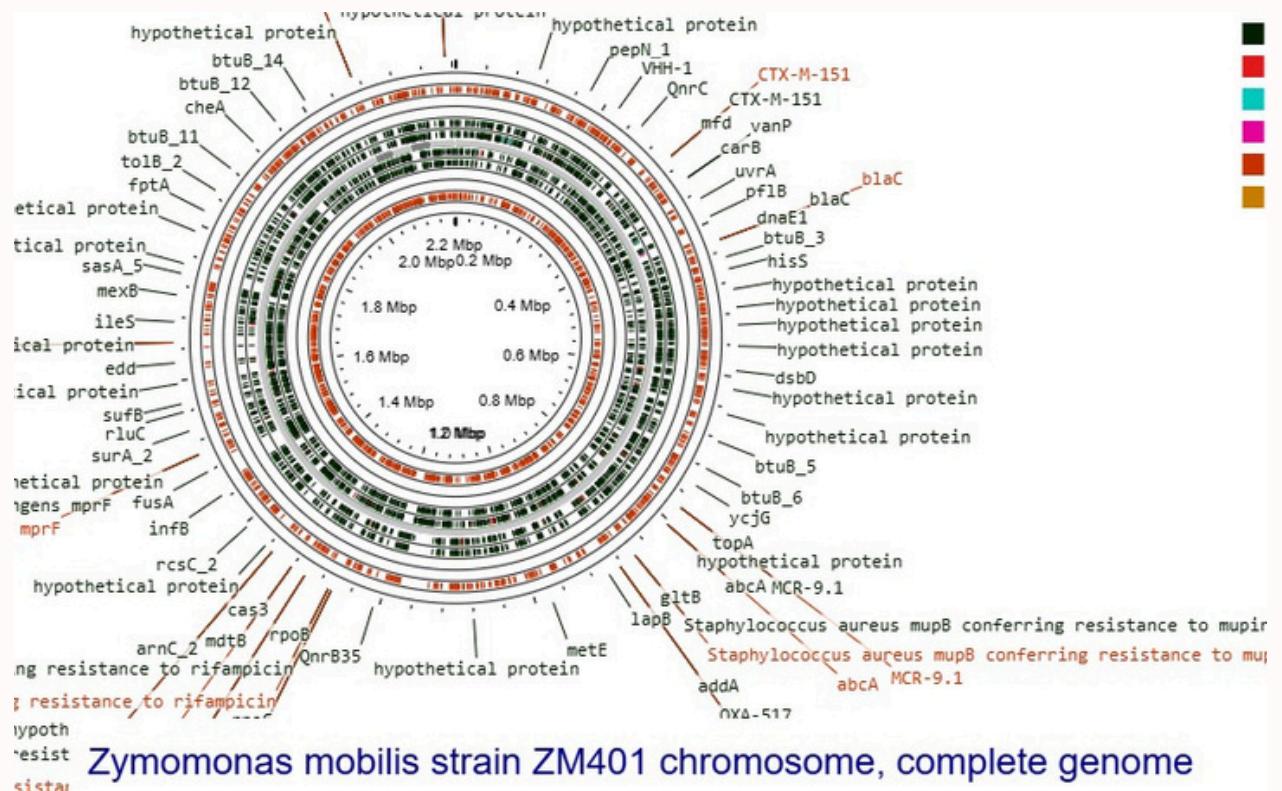


#### Common AMR Gene Family

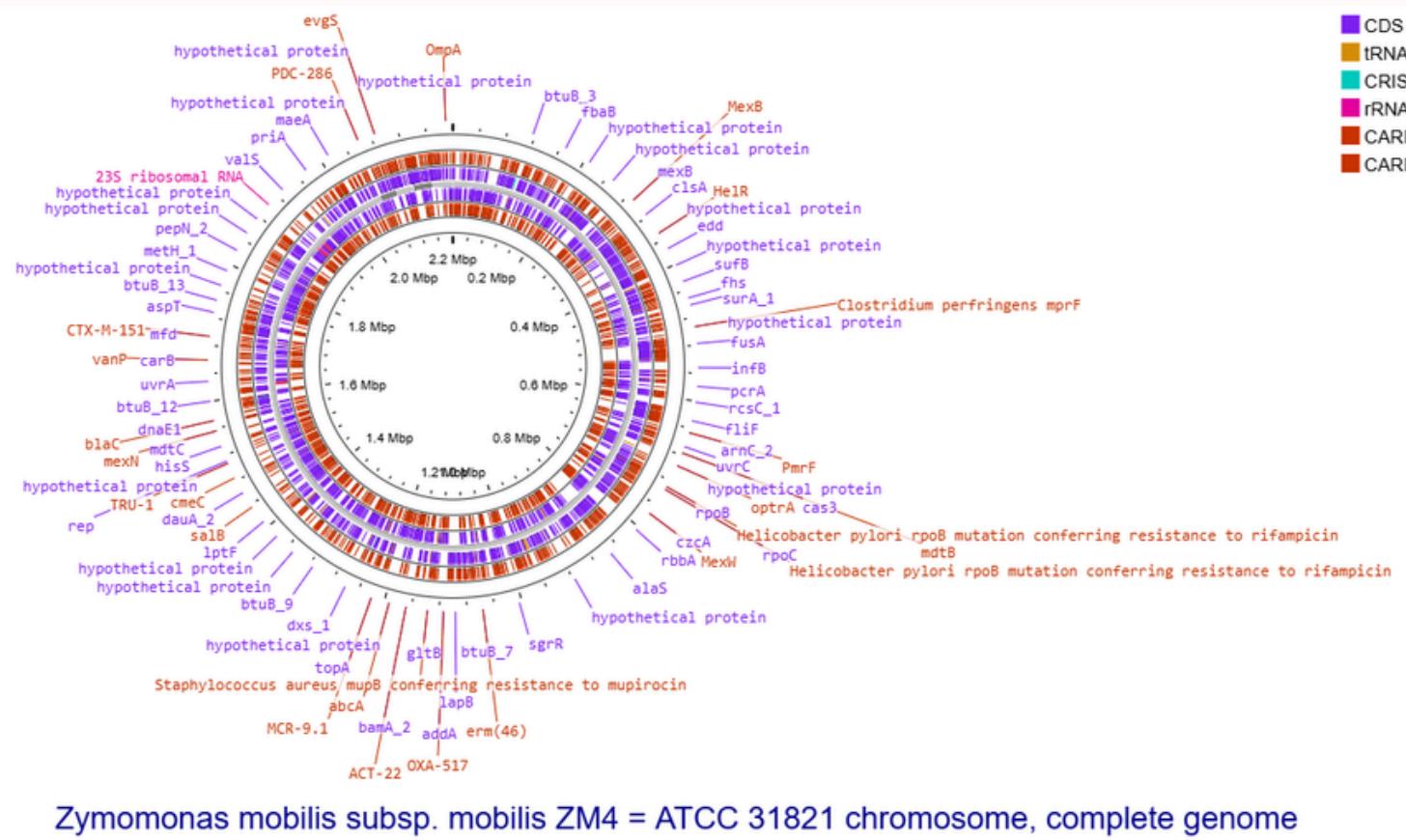
1. ACT beta-lactamase
2. antibiotic-resistant isoleucyl-tRNA synthetase (ileS)
3. ATP-binding cassette (ABC) antibiotic efflux pump
4. chloramphenicol acetyltransferase (CAT)
5. Erm 23S ribosomal RNA methyltransferase
6. glycopeptide resistance gene cluster; Van ligase
7. Miscellaneous ABC-F subfamily ATP-binding cassette ribosomal protection proteins
8. macrolide phosphotransferase (MPH)
9. major facilitator superfamily (MFS) antibiotic efflux pump
10. MCR phosphoethanolamine transferase
11. msr-type ABC-F protein
12. multidrug and toxic compound extrusion (MATE) transporter
13. OXA beta-lactamase
14. pmr phosphoethanolamine transferase
15. resistance-nodulation-cell division (RND) antibiotic efflux pump
16. sal-type ABC-F protein
17. vanH; glycopeptide resistance gene cluster
18. vga-type ABC-F protein

# Open Reading Frames of the species (Data from Proksee ORFs).

## *A. Zymomonas mobilis* ZM401 strain:

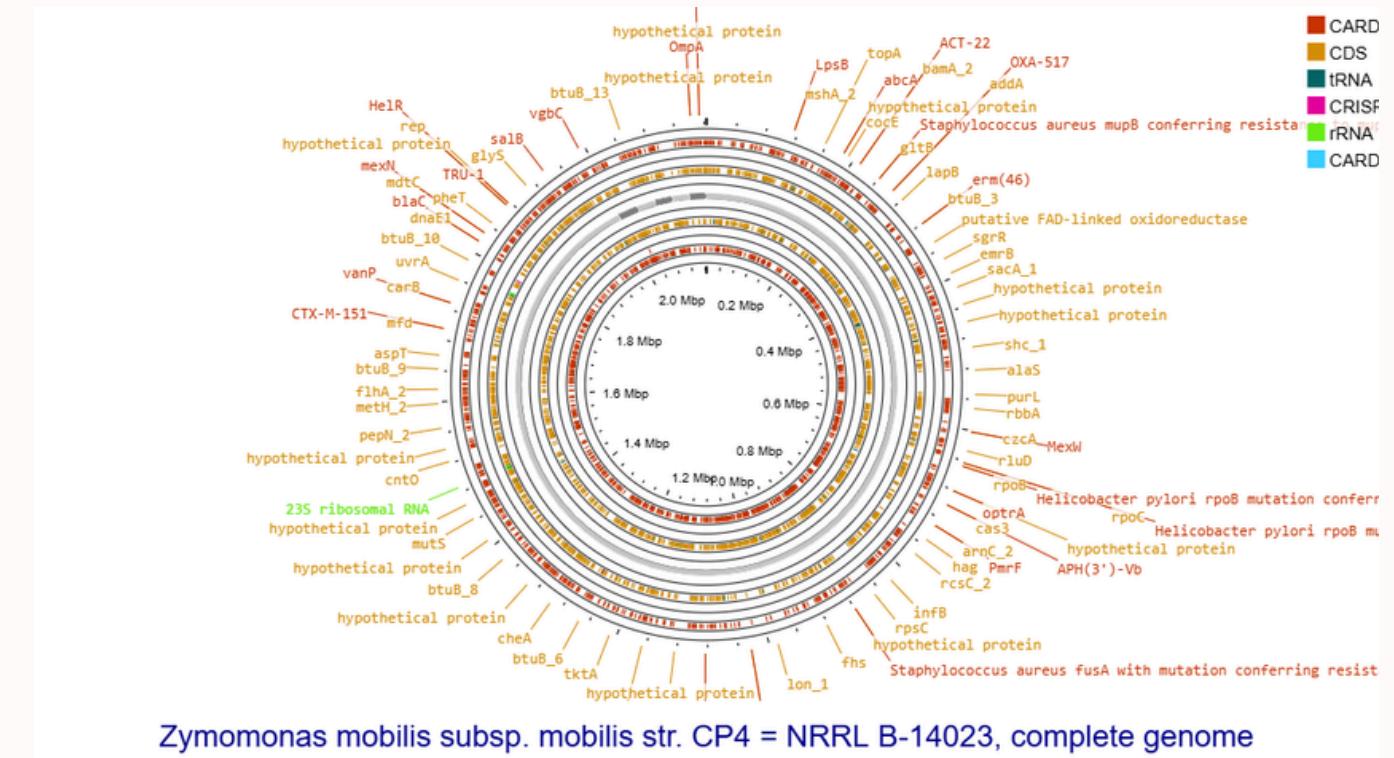


# **C. Zymomonas mobilis subsp. mobilis ZM4 = ATCC 31821**



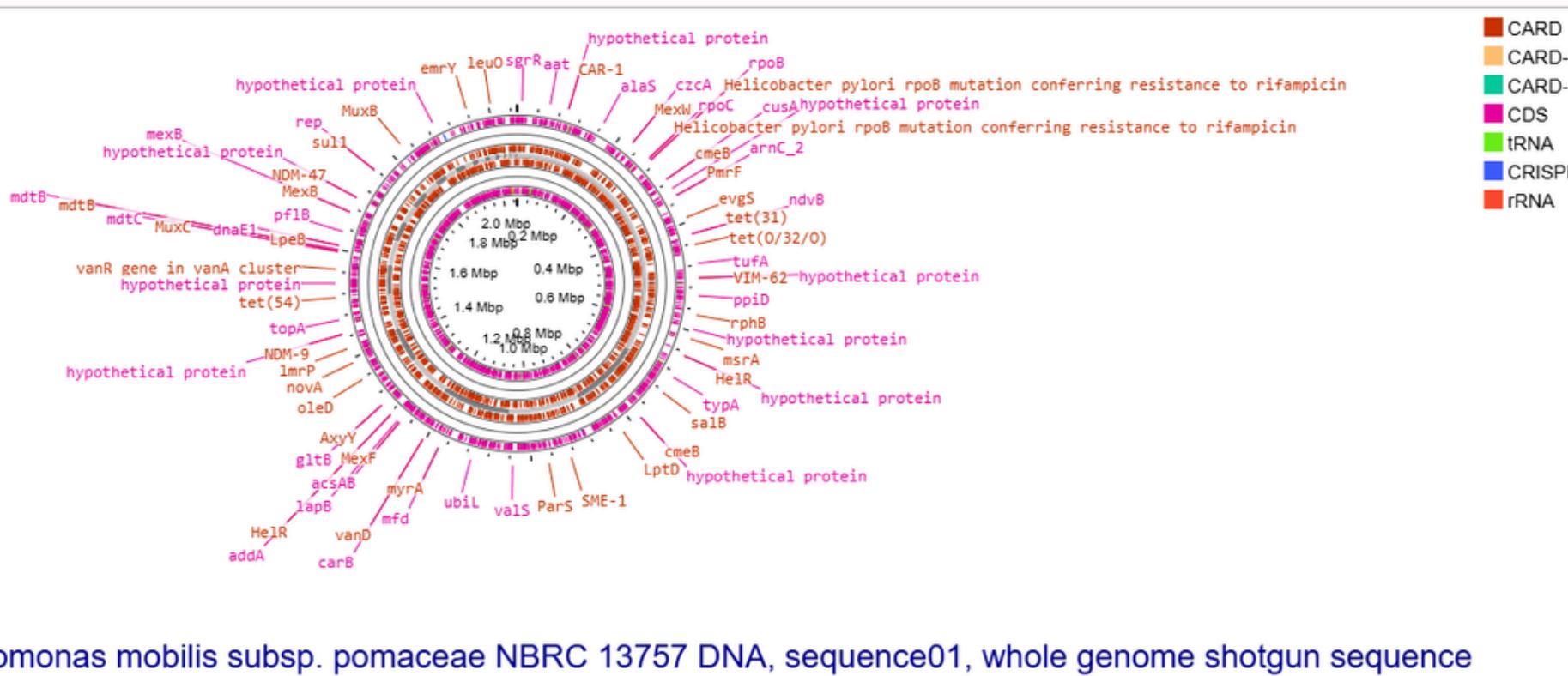
*Zymomonas mobilis* subsp. *mobilis* ZM4 = ATCC 31821 chromosome, complete genome

## **B. Zymomonas mobilis** CP4 strain:



*Zymomonas mobilis* subsp. *mobilis* str. CP4 = NRRL B-14023, complete genome

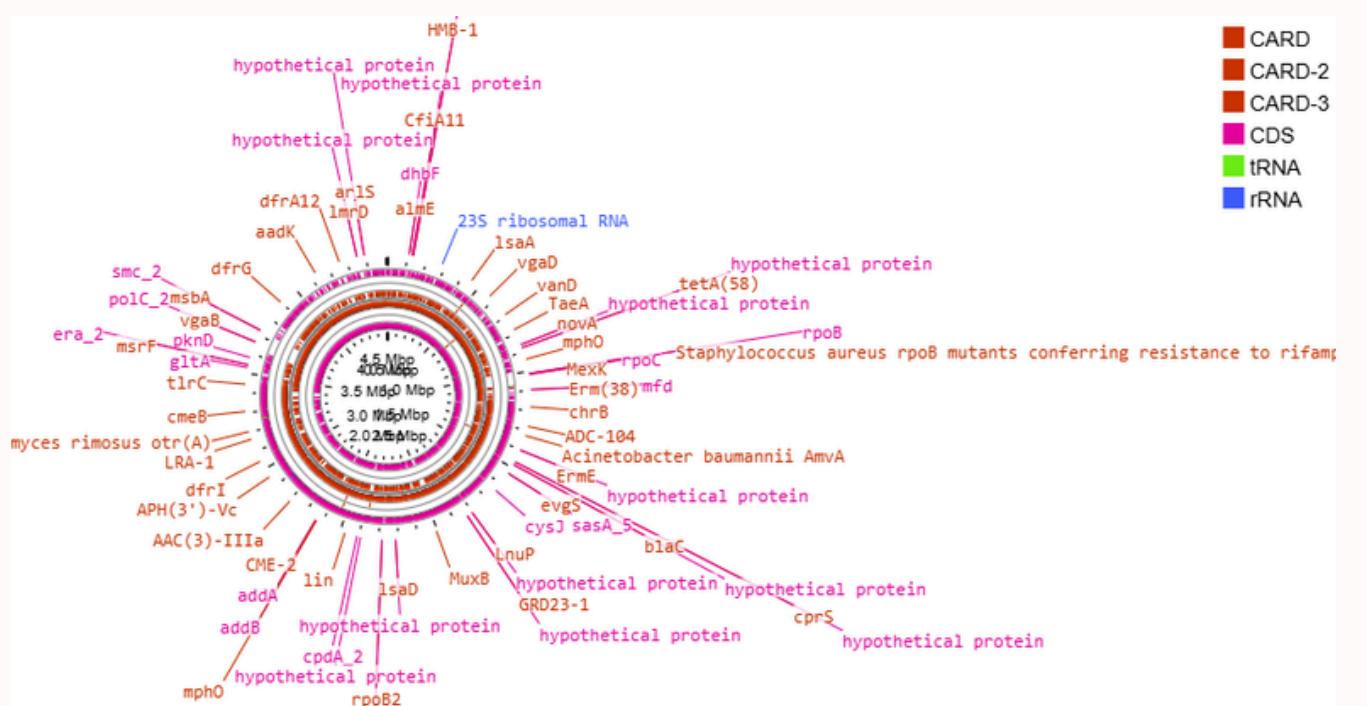
# D.*Zymomonas mobilis* subsp pomaceae:



*Zymomonas mobilis* subsp. *pomaceae* NBRC 13757 DNA, sequence01, whole genome shotgun sequence

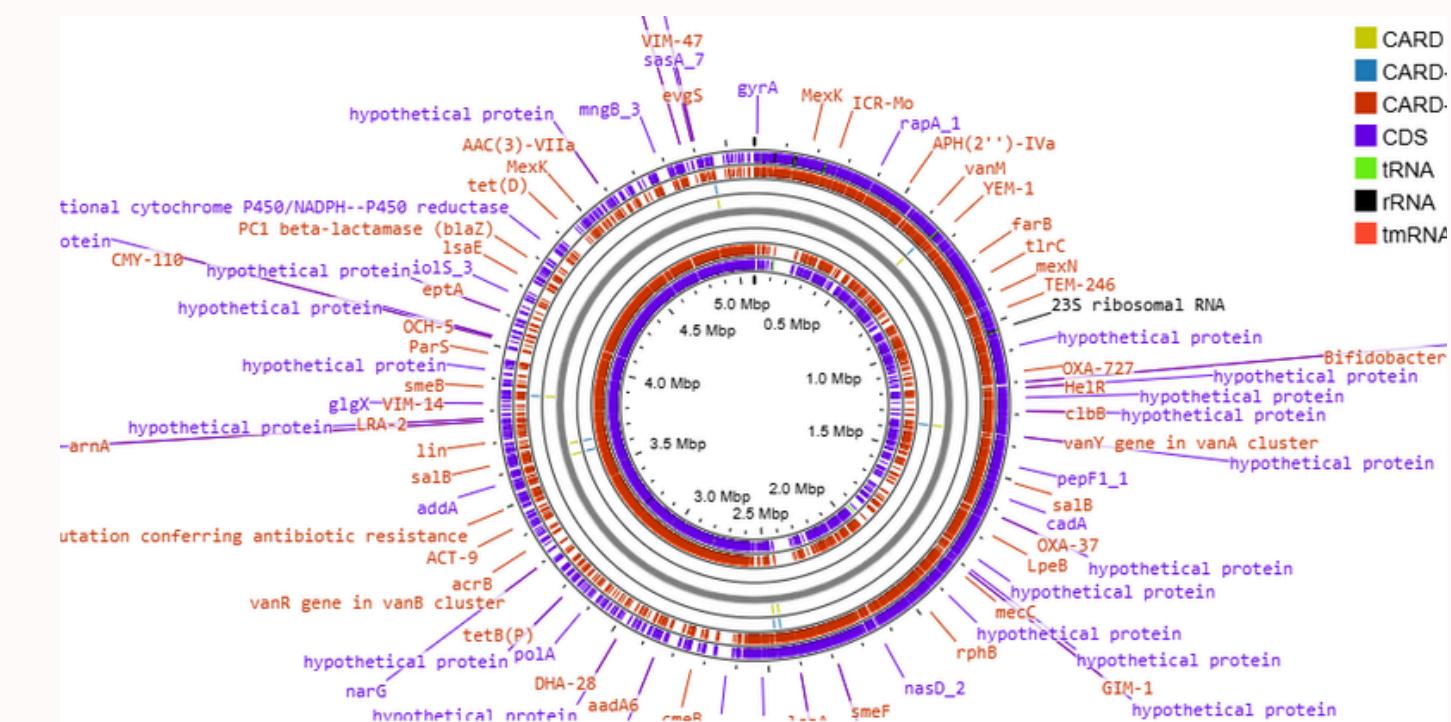
# Open Reading Frames of the species (Data from Proksee ORFs)

E.*Gracilibacillus caseinilyticus* strain: SSWR10-1



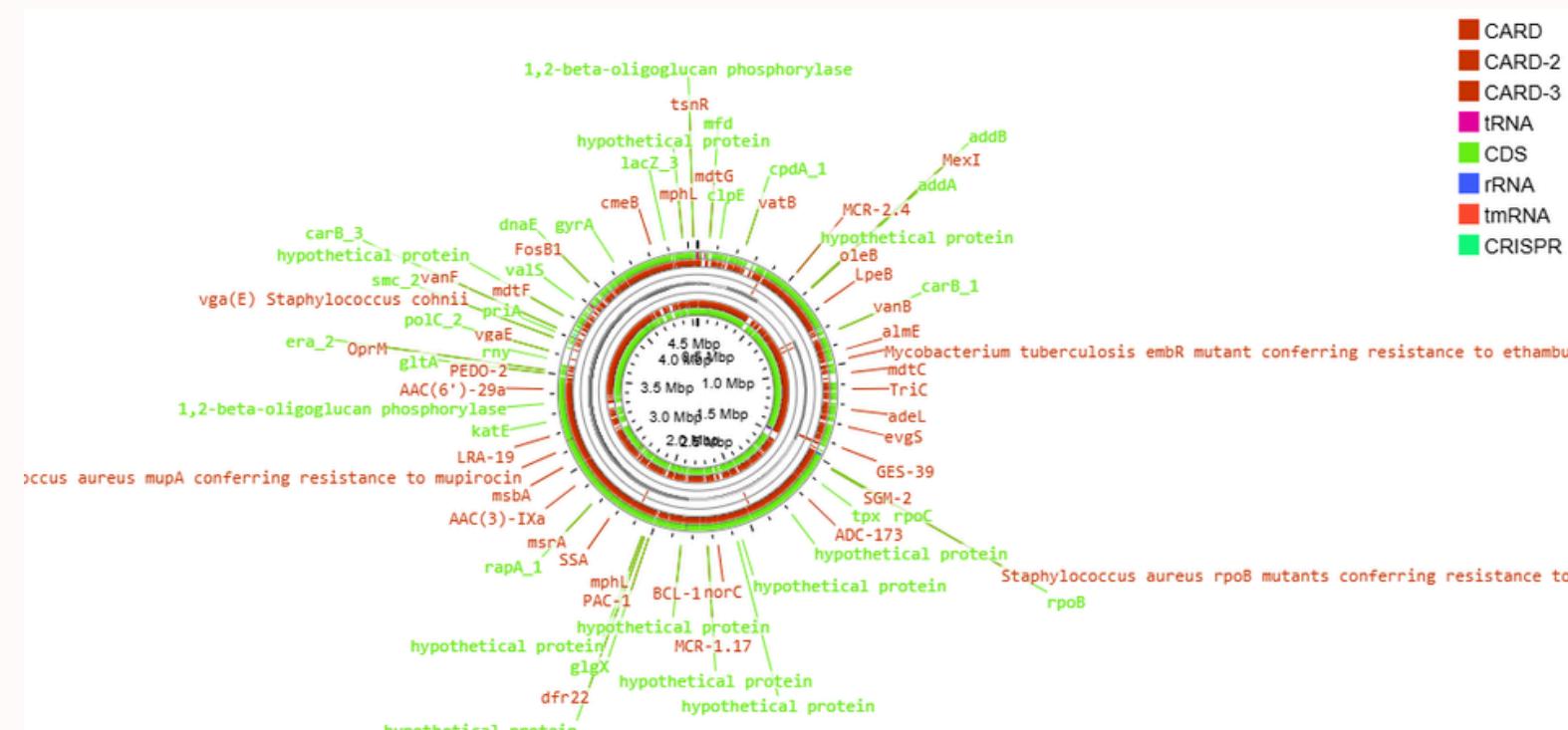
*Gracilibacillus caseinilyticus* strain SSWR10-1 chromosome, complete genome

F.*Gracilibacillus salitolerans* strain: SCU50



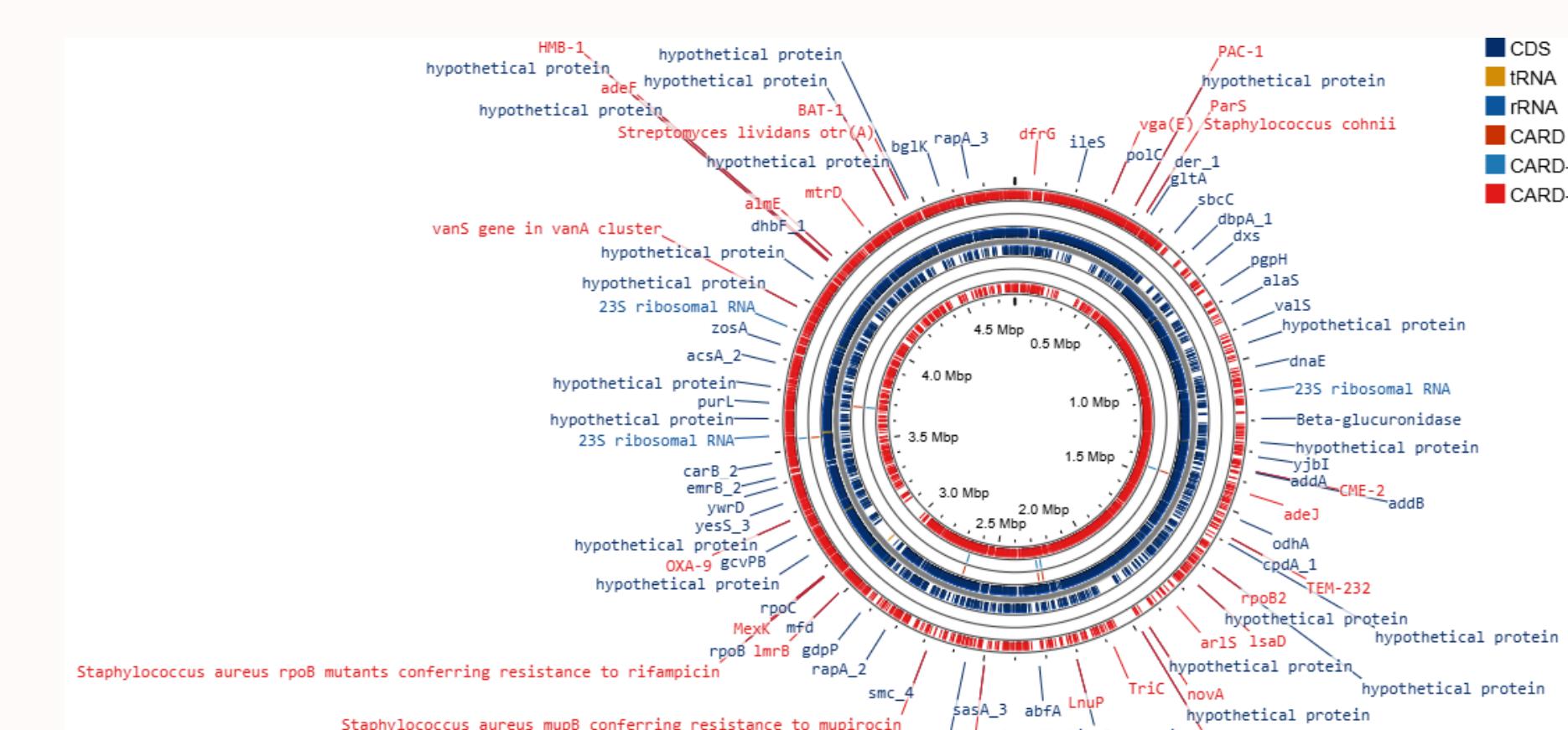
*Gracilibacillus salitolerans* strain SCU50 chromosome, complete genome

G. *Gracilibacillus sp. Marseille-P2481* strain: Marseille-P2481T



Marseille-P2481 strain Marseille-P2481T genome assembly, contig: contig00001, whole genome shc

H.*Gracilibacillus salinarum* strain: SSPM10-3

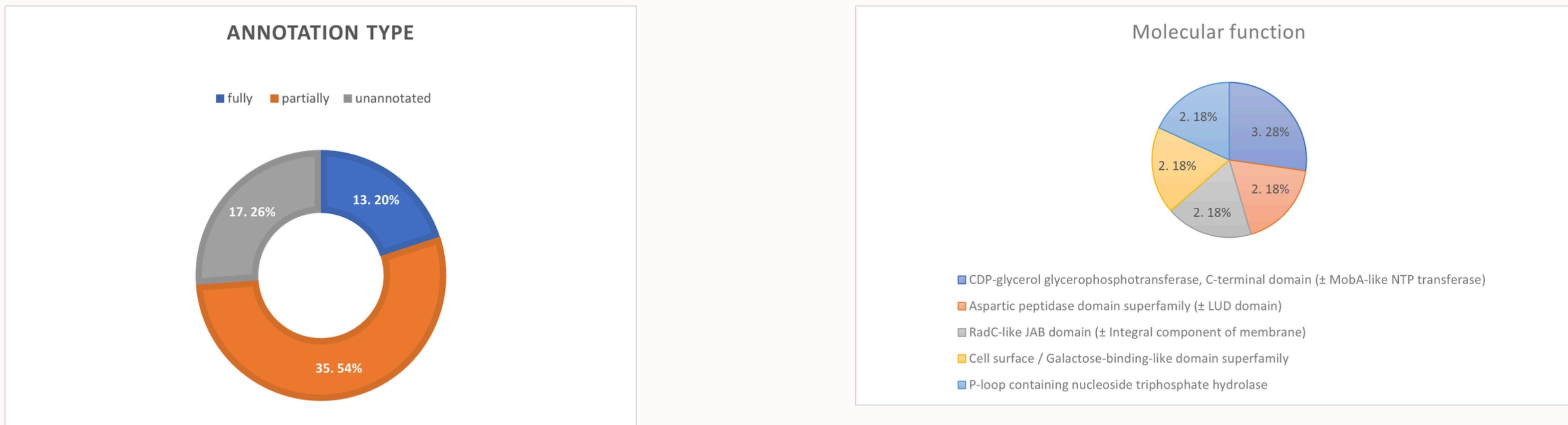


# Annotation of Hypothetical Proteins

## a. Hypothetical Proteins *Zymomonas mobilis* ZM401 strain:

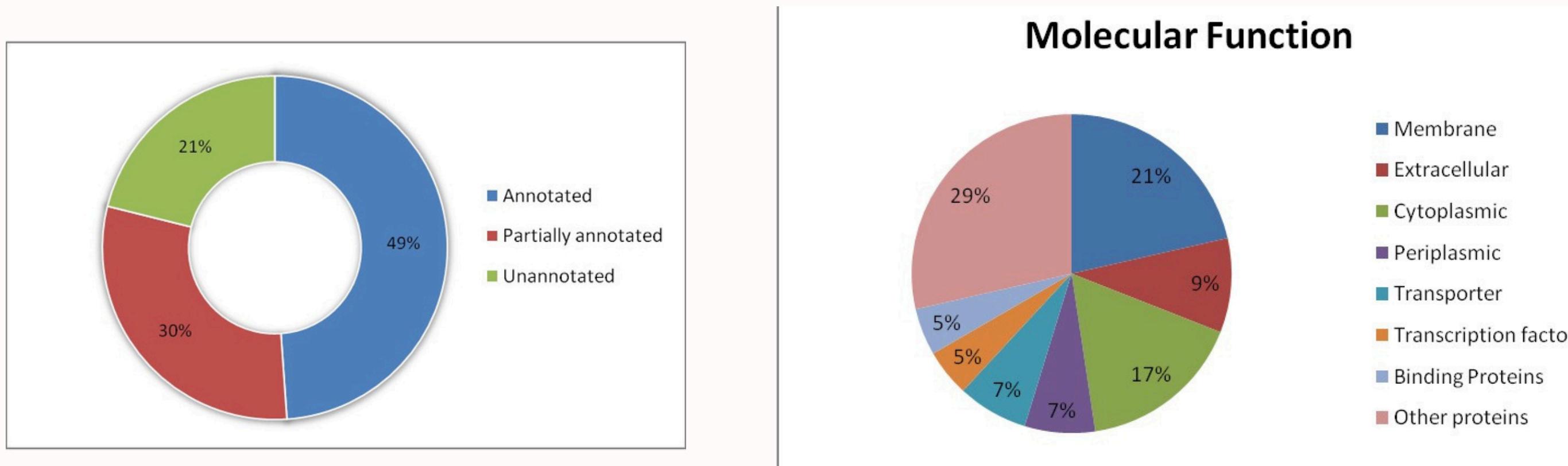


## b. Hypothetical Proteins *Zymomonas mobilis* CP4 strain:

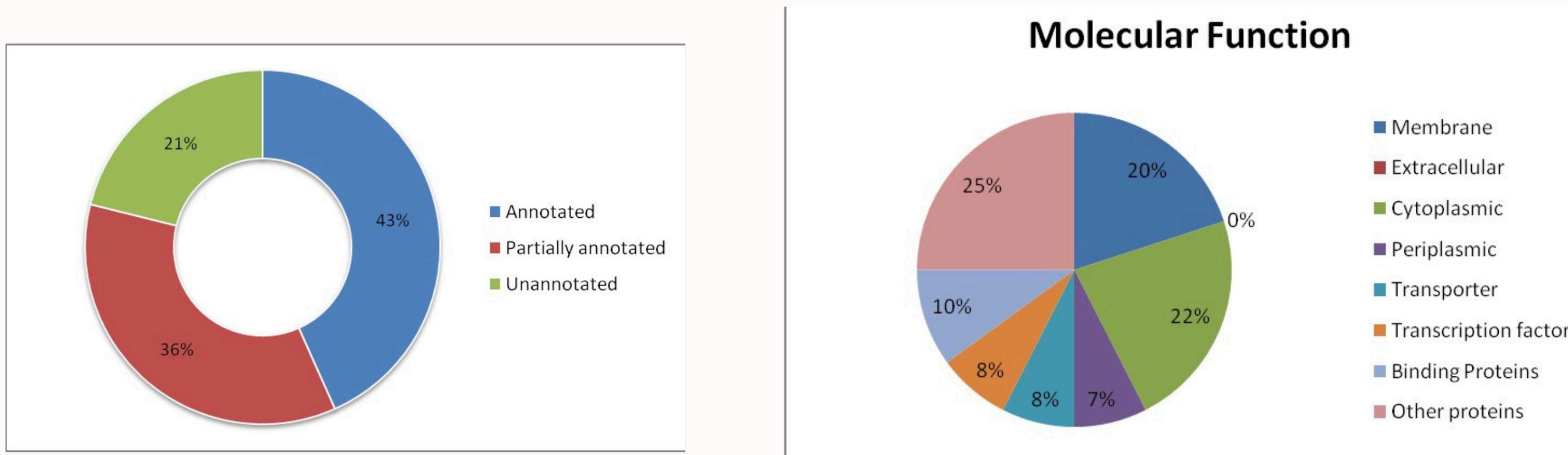


# Annotation of Hypothetical Proteins

## C. Hypothetical Proteins *Zymomonas mobilis* subsp. *mobilis* ZM4 = ATCC 31821n:

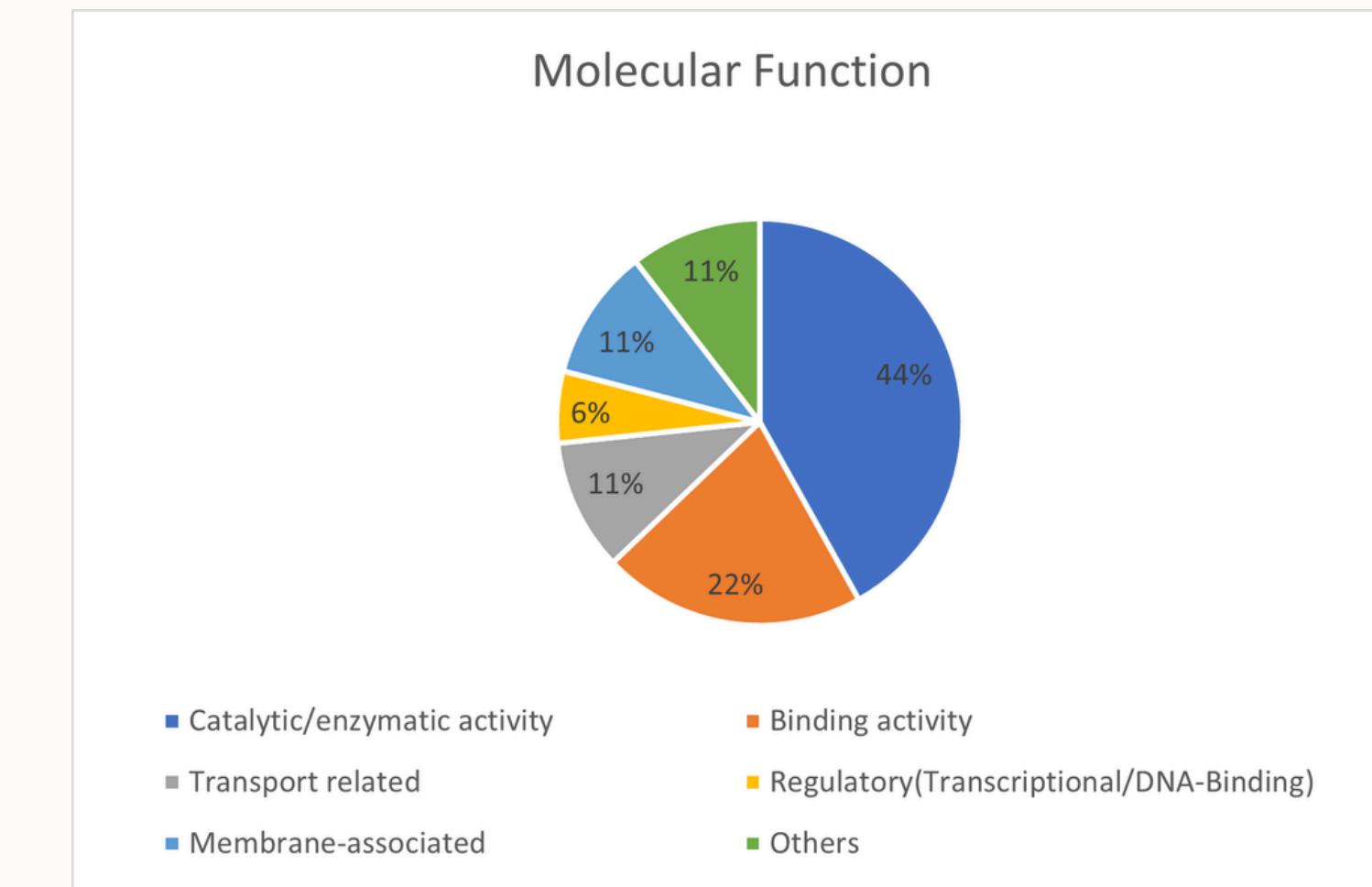
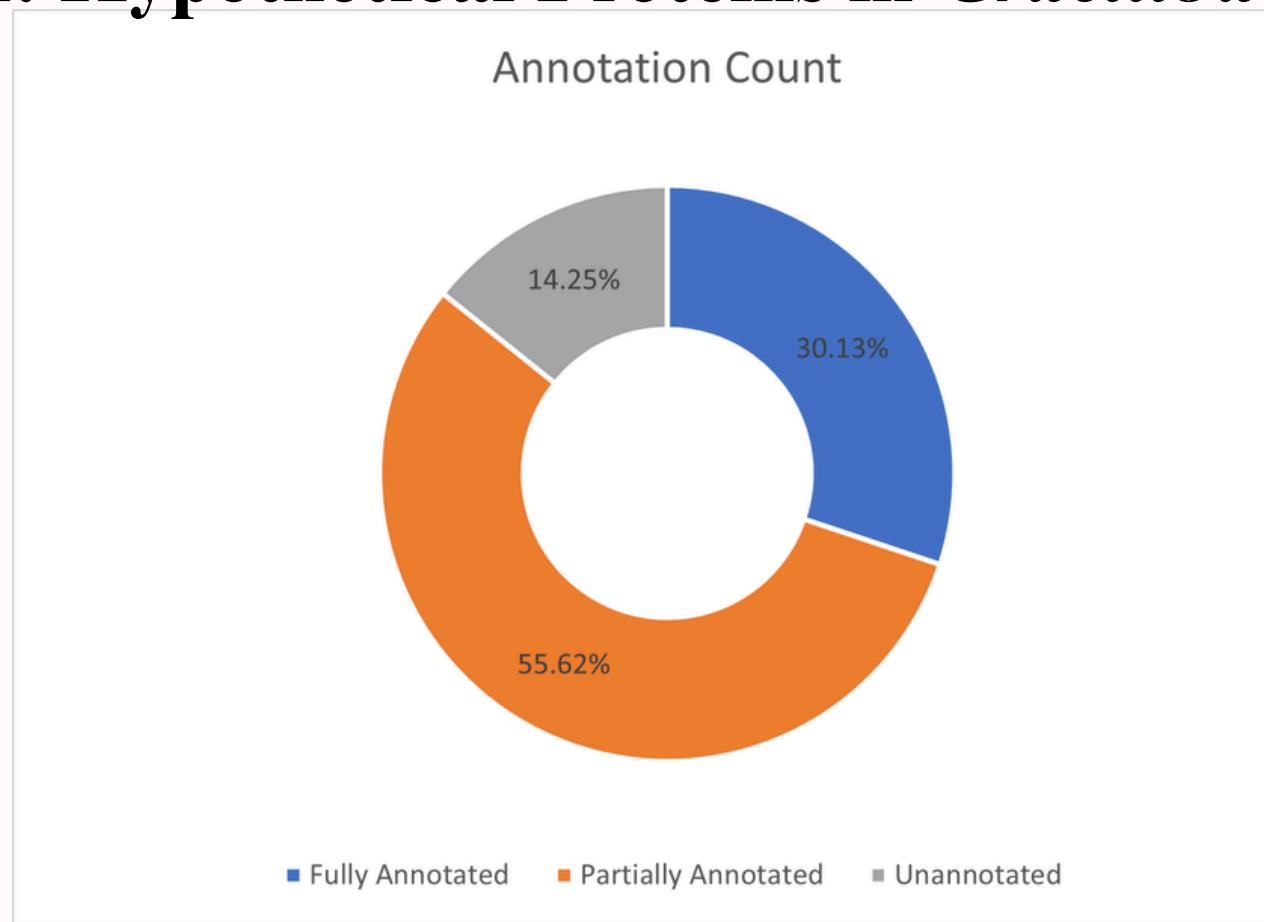


## D. Hypothetical Proteins *Zymomonas mobilis* subsp. *pomaceae*:

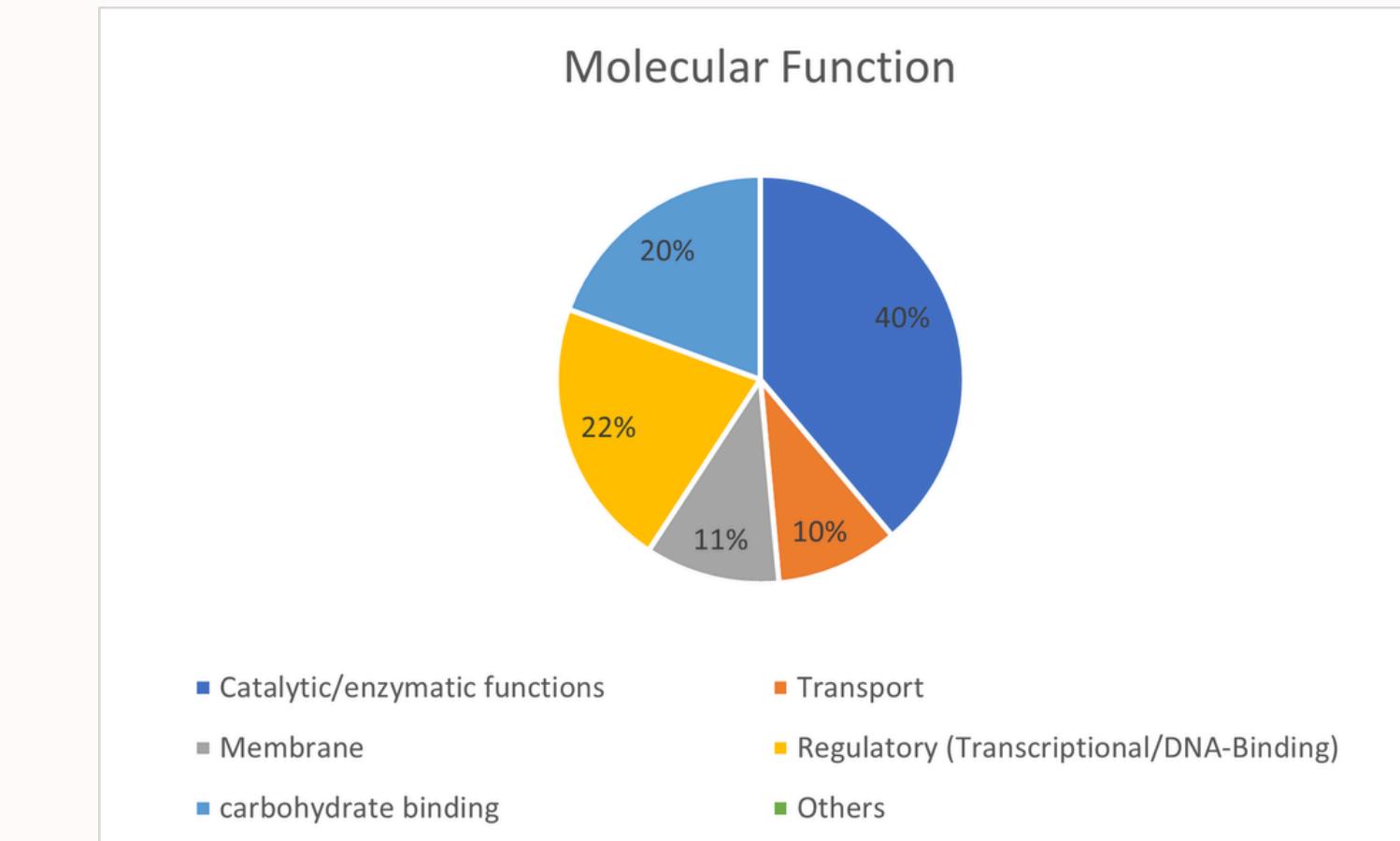
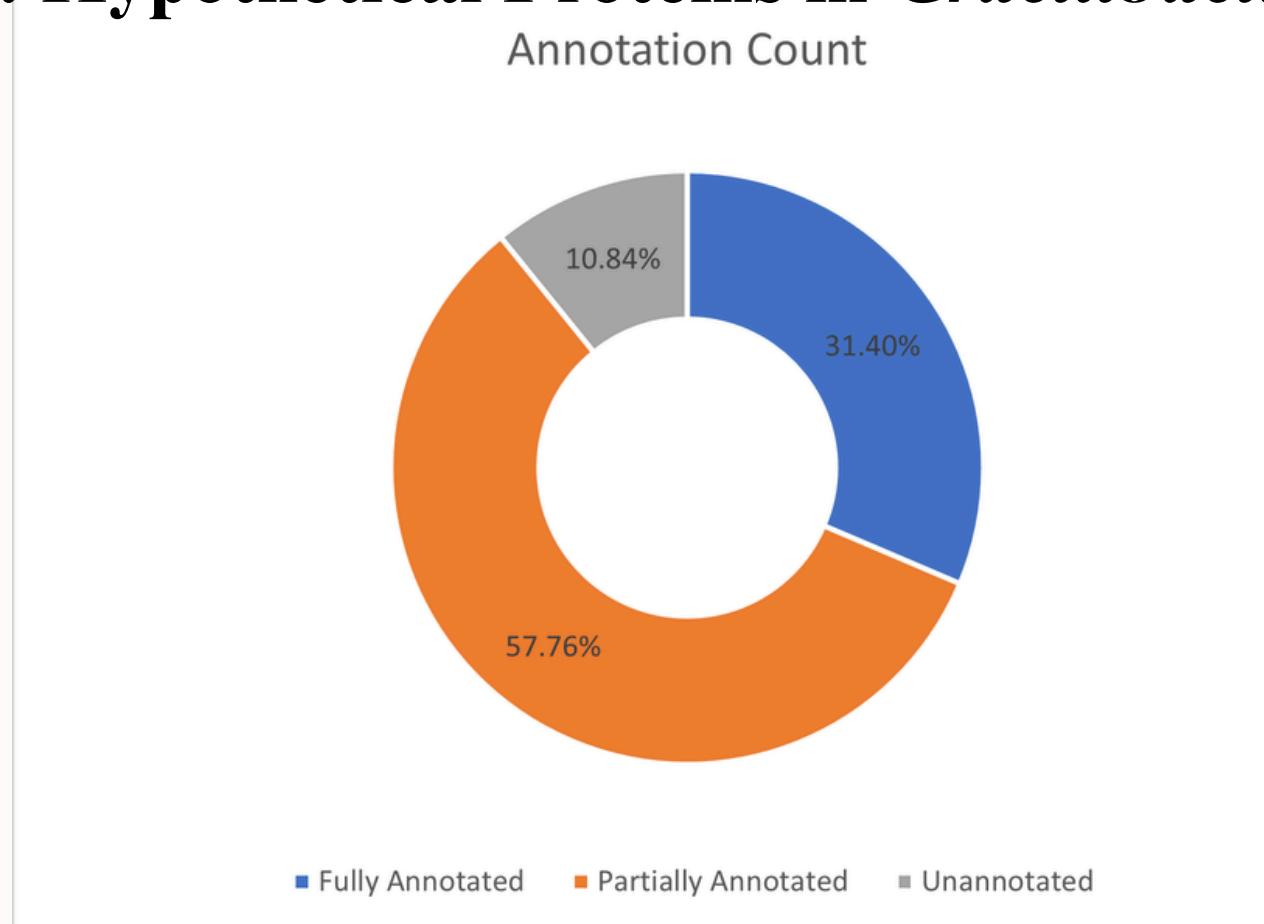


# Annotation of Hypothetical Proteins

## E. Hypothetical Proteins in *Gracilibacillus salitolerans*:

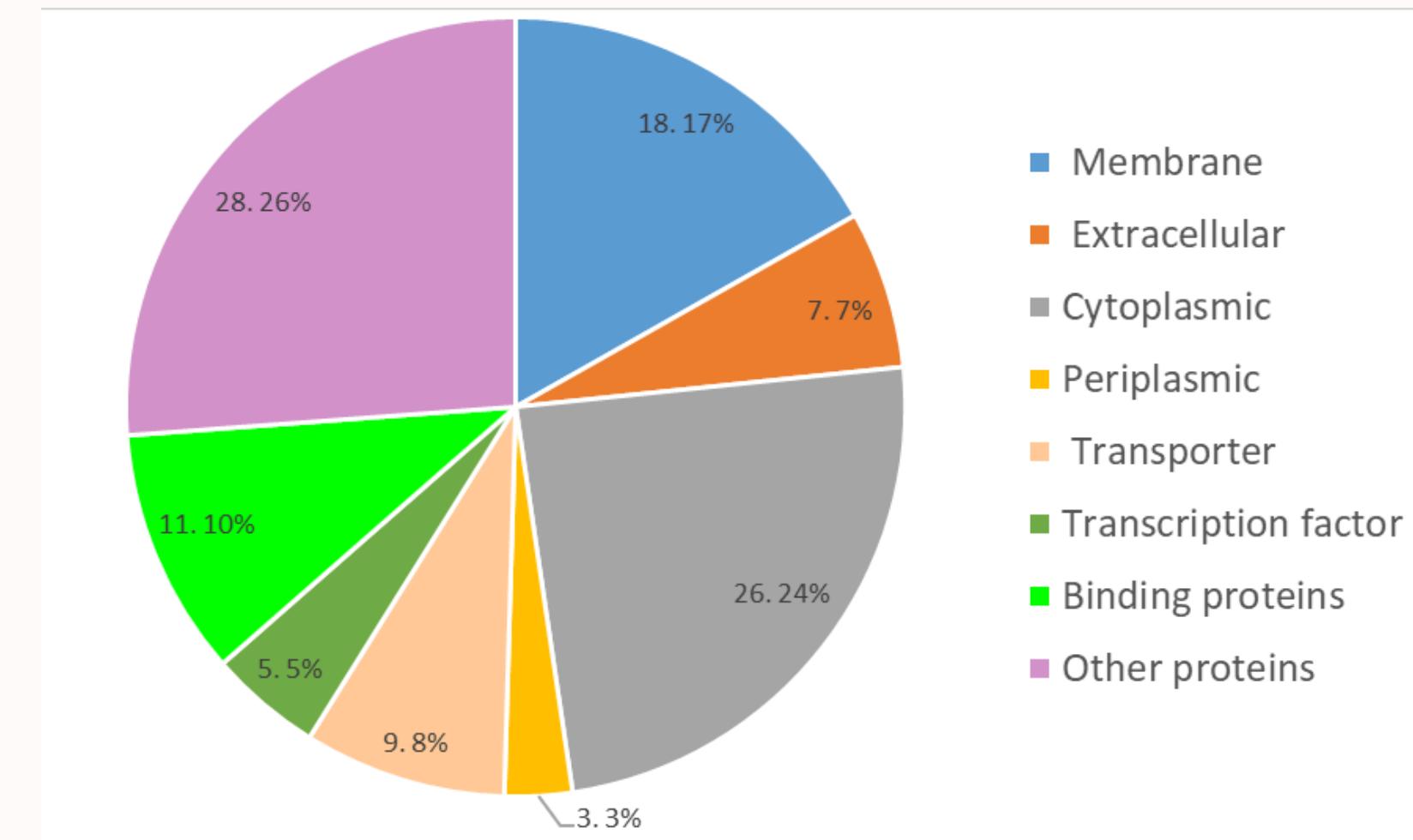
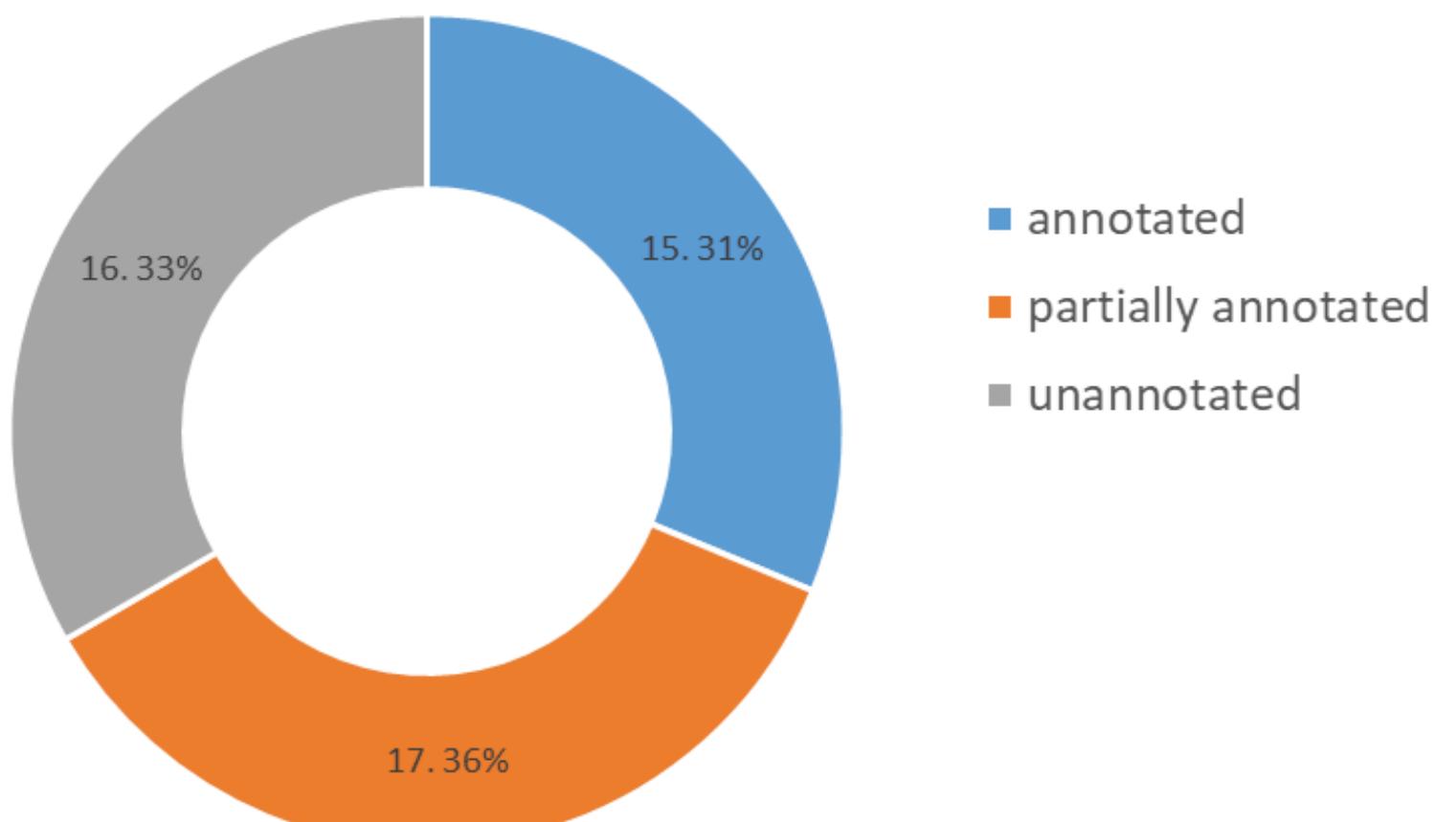


## F. Hypothetical Proteins in *Gracilibacillus caseinilyticus*:

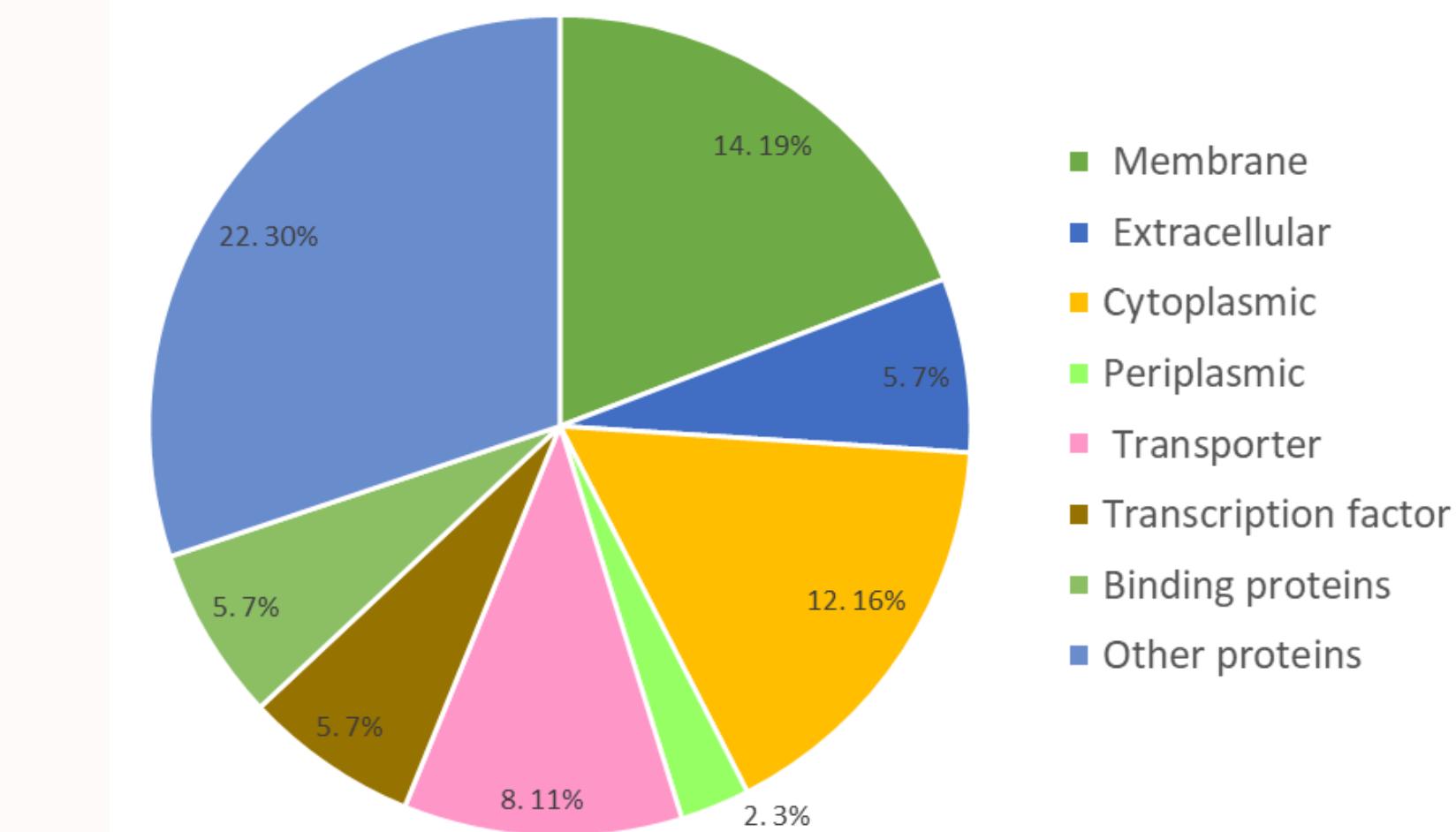
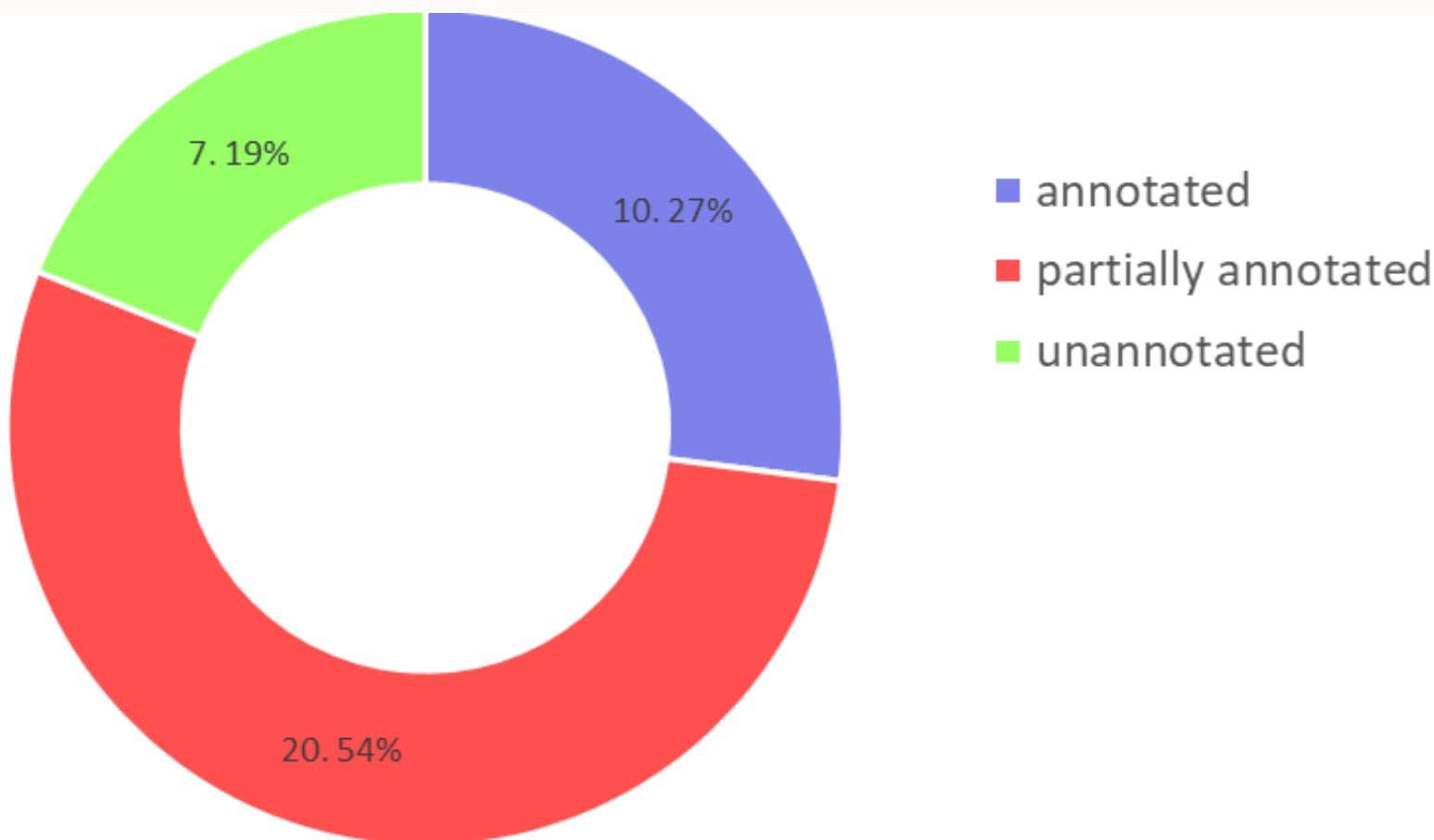


# Annotation of Hypothetical Proteins

## G. Hypothetical Proteins in *Gracilibacillus salinarum* :



## H. Hypothetical Proteins in *Gracilibacillus marseille* :



# KEY FINDINGS

## *Zymomonas mobilis*

### 1. Robust Multidrug Efflux Systems are a Primary Defense Mechanism

- RND Transporters are a major player, showing a 7-fold upregulation under inhibitor stress to pump out toxic compounds like vanillin and furfural.
- ABC Transporters are also crucial, showing 3-fold upregulation for expelling toxins and maintaining homeostasis.
- Specific efflux pump genes identified include ZMO0282, ZMO0283, and ZMO0285 (forming an RND operon). Knocking out this operon improved tolerance, proving its active role in transporting harmful substances.

### 2. Master Regulators Control the Stress Response

- The TetR family of transcription factors is upregulated under stress and directly controls the expression of efflux pumps like ABC transporters.
- The MarR family, a well-known antibiotic resistance regulator in other bacteria, is active in *Z. mobilis* and controls "multi drug efflux pumps."
- The global regulator Hfq (an RNA chaperone) is essential for optimal growth under stress. Overexpressing hfg enhances tolerance by coordinating the stress response at a post-transcriptional level.

### 3. The Cell Membrane is Dynamically Reinforced

- *Z. mobilis* decreases the unsaturated/saturated fatty acid ratio in its membrane, making it less fluid and less permeable to toxic molecules.
- Hopanoids (sterol-like compounds) play a key role in membrane stability, with their biosynthesis genes (e.g., hnpA, hnpB) upregulated under ethanol stress.

# **KEY FINDINGS**

## ***Gracilibacillus:***

### **1. Salt Stress Enriches Specific Antibiotic Resistance Genes (ARGs)**

- Soil salinity significantly increases the abundance and alters the profile of ARGs.
- The most enriched resistance mechanisms are antibiotic efflux (e.g., RND, ABC transporters) and target protection (e.g., rRNA methyltransferase).
- This leads to the evolution of multidrug resistance in soil microbes.

### **2. Mobile Genetic Elements (MGEs) Drive the Spread**

- Salt stress increases the abundance of MGEs like transposons, integrons, and plasmids.
- A high degree of genetic linkage (synteny) is found between ARGs and MGEs on the same DNA contigs, facilitating Horizontal Gene Transfer (HGT).
- Salt stress also induces physiological changes (oxidative stress, SOS response) that further promote HGT.

### **3. Microbial Community Shift Favors ARG Carriers**

- Salinity alters the soil bacterial community, enriching for phyla like Actinobacteria.
- ARG propagation occurs through both Vertical Gene Transfer (growth of resistant hosts) and Horizontal Gene Transfer.

### **4. Core Finding: Co-selection of Salt and Antibiotic Resistance**

- There is a direct genetic linkage between salt-tolerance genes (e.g., for K<sup>+</sup> uptake and organic osmolyte transporters) and ARGs on the same genomic contigs.
- This creates a co-selection pressure: when bacteria evolve to tolerate salt, they simultaneously select for linked antibiotic resistance genes through co-resistance and cross-resistance mechanisms.

# DISCUSSIONS

## Resistance mechanism

Bacteria gain antibiotic resistance through a combination of intrinsic evolutionary adaptations and horizontal acquisition of genetic elements. Genomic analysis of *Zymomonas* and *Gracilibacillus* strains reveals that environmental bacteria naturally possess diverse resistance mechanisms—including antibiotic efflux, target modification, and enzymatic inactivation—without clinical antibiotic exposure. While *Zymomonas* exhibits graded resistance across strains, *Gracilibacillus* shows a binary pattern, indicating niche-specific evolution. Crucially, resistance genes are often located on mobile genetic elements, facilitating horizontal transfer between environmental and pathogenic bacteria.

### **Similarities:**

1. Universal Presence of Efflux Mechanisms - All resistant strains across both genera utilize antibiotic efflux as a core resistance strategy
2. Multi-mechanism Approach - Resistant strains employ combinations of 5-6 different resistance mechanisms
3. Environmental Origin - All resistance mechanisms appear intrinsic rather than acquired through clinical exposure

### **Dissimilarities:**

1. Distribution Pattern:
  - *Zymomonas*: Graded distribution
  - *Gracilibacillus*: similar distribution
2. Mechanism Complexity:
  - *Zymomonas pomaceae* shows combined mechanisms (e.g., "target alteration + efflux")
  - *Gracilibacillus* resistant strains show identical, comprehensive 6-mechanism profiles

The most critical finding is that antibiotic resistance potential is highly strain-specific within *Zymomonas mobilis*. Strains like subsp. *pomaceae* are particularly noteworthy as "resistance reservoirs." Possessing 9 different mechanisms, including combinations, makes them a significant potential source for resistance gene transfer. While clear dichotomy suggests that resistance is not a random trait but is critically important for survival in the specific ecological niches of *G. caseinolyticus* and *G. marselle*. In contrast, *G. salitolerans* and *G. salinarum* may inhabit environments with lower antibiotic pressure. *G. caseinolyticus* and *G. marselle* are highly significant as environmental reservoirs for multi-drug resistance. Their ability to resist antibiotics via all possible mechanisms makes them a potential source of highly versatile resistance genes that could be transferred to pathogens. This "all-or-nothing" pattern in *Gracilibacillus* is different from the more graded variation seen in *Zymomonas*, highlighting that evolutionary paths to building a resistome can vary dramatically between different bacterial genera.

# DISCUSSIONS

## Antibiotic resistance genes

### 1. Universal Presence of an Intrinsic Resistome:

- All strains, across both genera, possess antibiotic resistance genes (ARGs), confirming that ARGs are a fundamental, natural feature of environmental bacteria.

### 2. Striking Genus-Wide vs. Species-Specific Patterns:

- *Zymomonas mobilis*: Shows a consistent, core resistome. All four strains share a very similar profile of major resistance genes (e.g., efflux pumps, OXA, tetracycline resistance).
- *Gracilibacillus*: Shows a highly variable, species-specific resistome. Two species (*G. caseinolyticus* and *G. marseille*) are heavily armed with numerous ARGs, while the other two (*G. salitolerans* and *G. salinarum*) have very few.

### 3. Common High-Risk Resistance Mechanisms:

- Both genera carry genes for multi-drug efflux pumps (ABC, MFS, RND), highlighting this as a universal first-line defense.
- Critically important beta-lactamase genes (OXA) are common to strains in both genera.
- Resistance genes for tetracyclines (ribosomal protection, inactivation) and macrolides (Erm) are frequently found.

## Significance and Similarities

### 1. The Environment is a Primary Resistance Reservoir:

- The most critical finding is that potent, clinically relevant resistance genes (e.g., OXA beta-lactamase, Vancomycin clusters, MCR) pre-exist naturally in non-pathogenic environmental bacteria. They were not created by clinical antibiotic use.

### 2. High Potential for Horizontal Gene Transfer (HGT):

- The presence of identical, high-impact genes (like OXA) in such distantly related genera strongly suggests these genes are mobile. This means the resistome is a shared network, not a series of isolated events. Environmental bacteria like these are likely contributors to the ARGs that eventually emerge in human pathogens.

### 3. Contrasting Evolutionary Strategies:

- *Zymomonas* demonstrates a "core genome" strategy, where resistance is a built-in, conserved trait for the entire genus.
- *Gracilibacillus* demonstrates an "accessory genome" strategy, where resistance is a specialized, variable trait acquired by specific species, likely due to niche-specific pressures.
- This shows there are multiple evolutionary paths to building a resistome.

### 4. Direct Implication for One Health:

- These findings underscore the One Health principle—that human, animal, and environmental health are interconnected. Monitoring environmental bacteria is crucial for understanding and mitigating the global spread of antibiotic resistance.

Feature	<i>Gracilibacillus</i>	<i>Zymomonas</i>
Gram Reaction	Gram-positive	Gram-negative
Endospore Formation	Endospore-forming	Non-endospore-forming
Oxygen Requirement	Aerobic	Aerotolerant, facultative anaerobe
Catalase Activity	Catalase-positive	Catalase-positive
Oxidase Activity	Oxidase-negative	Not typically reported as oxidase-positive
Optimal Growth Conditions	Grows in moderate salt concentrations	pH > 3.4, temperature 25–30 °C, tolerates up to 10% ethanol
Carbon Source Utilization	Various organic compounds; adapted to saline ecosystems	Utilizes glucose, fructose, sucrose; cannot use maltose, maltotriose, lactose, or cellobiose
Major Metabolic Pathway	Typical aerobic respiration	Entner–Doudoroff (ED) pathway (instead of EMP glycolysis)
Special Metabolic Traits	Salt tolerance mechanisms for osmotic stress	High ethanol productivity, lacks full TCA cycle
Industrial/Applied Importance	Adaptation studies in saline microbiology and extremophile research	Industrial bioethanol production; spoilage of alcoholic beverages

# FUTURE PROSPECTIVES

## *Zymomonas mobilis*

### Abstract

Nowadays, biofuels, especially bioethanol, are becoming increasingly popular as an alternative to fossil fuels. *Zymomonas mobilis* is a desirable species for bioethanol production due to its unique characteristics, such as low biomass production and high-rate glucose metabolism. However, several factors can interfere with the fermentation process and hinder microbial activity, including lignocellulosic hydrolysate inhibitors, high temperatures, an osmotic environment, and high ethanol concentration. Overcoming these limitations is critical for effective bioethanol production. In this review, the stress response mechanisms of *Z. mobilis* are discussed in comparison to other ethanol-producing microbes. The mechanism of stress response is divided into physiological (changes in growth, metabolism, intracellular components, and cell membrane structures) and molecular (up and down-regulation of specific genes and elements of the regulatory system and their role in expression of specific proteins and control of metabolic fluxes) changes. Systemic metabolic engineering approaches, such as gene manipulation, overexpression, and silencing, are successful methods for building new metabolic pathways. Therefore, this review discusses systems metabolic engineering in conjunction with systems biology and synthetic biology as an important method for developing new strains with an effective response mechanism to fermentation stresses during bioethanol production. Overall, understanding the stress response mechanisms of *Z. mobilis* can lead to more efficient and effective bioethanol production.

**Keywords** Bioethanol fermentation stress condition, Metabolic engineering, Stress response regulatory network, Synthetic biology, Systems biology, *Zymomonas mobilis*

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REVIEW



### Perspectives and new directions for bioprocess optimization using *Zymomonas mobilis* in the ethanol production

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### Abstract

*Zymomonas mobilis* is an ethanogenic microbe that has a demonstrated potential for use in lignocellulosic biorefineries for bioethanol production. *Z. mobilis* exhibits a number of desirable characteristics for use as an ethanogenic microbe, with capabilities for metabolic engineering and bioprocess modification. Many advanced genetic tools, including mutation techniques, screening methods and genome editing have been successively performed to improve various *Z. mobilis* strains as potential consolidated ethanogenic microbes. Many bioprocess strategies have also been applied to this organism for bioethanol production. *Z. mobilis* biofilm reactors have been modified with various benefits, including high bacterial populations, less fermentation times, high productivity, high cell stability, resistance to the high concentration of substrates and toxicity, and higher product recovery. We suggest that *Z. mobilis* biofilm reactors could be used in bioethanol production using lignocellulosic substrates under batch, continuous and repeated batch processes.

**Keywords** Biofilm reactor • Ethanol • *Zymomonas mobilis* • Metabolic Engineering

The following points gives the overview of the possible future prospectives of using *Zymomonas mobilis*:

- Enhancing Feedstock Utilization and Range by substrate spectrum expansion and consolidated bioprocessing- The main goal is to achieve complete utilization of renewable, non-foid competing feedstock to maximize biorefinery economic viability and significantly reducing the capital and operating cost
- Strain Engineering and Tolerance Mechanisms by increasing Tolerance and Robustness with the help of advance genetic tools and accelerated strain improvement.
- Process Optimization and Bioproduction Diversification- Expanding production capabilities to compounds such as 2,3-butanediol, sorbitol, and succinic acid, diversifying the biorefinery output. The ultimate goal being applying these advancements toward process optimization, develop predictive scale-up models, and design comprehensive, cost-effective biorefineries that harness the organism's unique metabolic efficiencies.

# FUTURE PROSPECTIVES

## *Gracilibacillus spp.*

### 1. Antibiotic Resistance and Salt Stress

- Salt stress increases the abundance of antibiotic resistance genes (ARGs) in saline environments.
- Major resistance mechanisms include efflux pumps (RND, ABC transporters) and rRNA methyltransferases.
- Mobile genetic elements (plasmids, transposons, integrons) carry both salt-tolerance and resistance genes, leading to co-selection and multidrug resistance.

### 2. Key Research Directions

- Identification and characterization of plasmids that link salt-tolerance and ARGs.
- Study of their genetic cargo, stability, and potential transfer to pathogens.
- Examination of how salinity, nutrients, or low antibiotic levels affect gene expression and plasmid conjugation.
- Ecological surveys in saline soils, hypersaline lakes, and food processing areas can be conducted to track ARG distribution.

### 3. Biotechnological Potential

- It is the source of halo-tolerant enzymes (proteases, lipases, amylases) for detergents and food industries.
- It is useful for biofuel production (biohydrogen, biobutanol) in high-salt, non-sterile conditions.
- Can help in bioremediation of saline or metal-contaminated wastewater.
- Acts as a plant growth-promoting bacterium (PGPR) in saline soils.

### 4. Scientific Importance

- Model organism for studying plasmid stability and gene transfer under salt stress.
- Potential chassis for synthetic biology in saline environments.
- Understanding its conjugation machinery can help limit ARG spread.

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