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Characterization of operons for sucrose metabolism in marine bacteria of the *Vibrio* genus

UNIVERSIDADE DO ALGARVE
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Trabalho realizado sob a orientação da Professora Doutora Deborah M Power e
Professor Doutor João CR Cardoso

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Characterization of operons for sucrose metabolism in marine bacteria of the *Vibrio* genus

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

I hereby declare to be the author of this work, which is original and unpublished. Authors and works consulted are properly cited in the text and included in the reference list.

Saeideh Eslami

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Abstract

Some species of the *Vibrio* genus are within the most frightening pathogenic bacteria to aquaculture and human health and the frequent emergence of novel bacteria strains represents an additional risk. The bacterium *Vibrio ichthyoenteri* is an important fish pathogen causing high mortality in aquaculture. Recently our group characterized two *V. ichthyoenteri isolates* (*V. ichthyoenteri* 1 and 2*)* and biochemical studies revealed that they have different phenotypes in relation to sucrose metabolism. The aim of this study was to understand the basis of their genetic and phenotypic differences and how this potentially relates with virulence using bioinformatics, microbiology, and molecular approaches. Bioinformatic annotation revealed that *V. ichthyoenteri* 1 has 4711 genes while *V. ichthyoenteri* 2 contains 4643 genes and the main differences reside in genes involved in functional/metabolic processes. Characterization of the sucrose operon revealed that two clusters (1A and 1B) exist in *V. ichthyoenteri* 1 (non-sucrose fermenter strain) while only one operon is present in *V. ichthyoenteri* 2 (sucrose fermenter strain). Like other bacteria the *V. ichthyoenteri sucrose* operon is composed of four genes, but sequence comparisons and phylogenetic analysis revealed that *V. ichthyoenteri* 1A is more like other Vibrios while *V. ichthyoenteri 1*B and *2* are identical. Gene linkage analysis revealed that *V. ichthyoenteri* 1A was likely to have acquired extra gene copies by horizontal gene transfer. The presence of sucrose did not modify bacteria growth kinetics but decreased the activity of enzymes that are potentially related with virulence. This was also true for the pathogenic *V. harveyi*. Gene expression studies targeting the sucrose hydrolysis gene (*scrB*) revealed that in both strains this gene is expressed in the presence or absence of sucrose and expression increases with culture time suggesting it was probably involved in other metabolic processes. The mechanism responsible for the difference in sucrose utilization between the two strains remains to be further explored.

Keywords: *Vibrio ichthyoenteri,* vibriosis, aquaculture, sucrose metabolism, sucrose operon

Resumo

As bactérias do género *Vibrio* ganharam notoriedade por serem alguns dos microorganismos patogénicos mais preocupantes em ambientes marinhos. Elas representam ameaças significativas tanto para a aquacultura quanto para as populações humanas. A incidência de surtos de vibriose está em ascensão, e o surgimento de novas estirpes de *Vibrio* agrava ainda mais os riscos que elas representam. Portanto, é imperativo obter uma compreensão profunda do comportamento das espécies de *Vibrio*, das bases genéticas, do potencial patogénico e dos riscos de infeção associados. As espécies de *Vibrio* são motivo de preocupação devido à sua alta abundância, biomassa substancial, taxas de crescimento rápido e capacidade de adaptação a ambientes diversos. Essas características as tornam fontes primárias de doenças infecciosas em ambientes aquáticos. Entre essas espécies, *V. harveyi, V. vulnificus, V. alginolyticus* e *V. parahaemolyticus* estão comumente associadas a surtos de vibriose, causando perdas econômicas significativas na indústria da aquacultura. Além disso, 12 espécies de *Vibrio* são reconhecidas como patógenos humanos, com destaque para *V. alginolyticus, V. cholerae, V. parahaemolyticus* e *V. vulnificus*. A exposição humana ao *Vibrio* geralmente ocorre por meio de água contaminada, consumo de frutos do mar crus ou mal cozidos ou contato de ferimentos com águas contaminadas. Além disso, as mudanças climáticas servem como uma variável adicional que afeta o comportamento, prevalência e virulência de patógenos marinhos. Projeções de modelos climáticos indicam um aumento esperado de temperatura de 2,5 a 10 °C no próximo século, uma mudança que pode proporcionar condições favoráveis para o aumento do crescimento de estirpes patogénicas de *Vibrio*.

Este estudo teve como objetivo identificar e caracterizar dois isolados de estirpes de *V. ichthyoenteri* encontrados na água dos tanques de solha senegalesa (Solea senegalensis) cultivada em instalações internas de Sistema de Aquacultura em Recirculação (RAS). O ponto de partida para esta extensa exploração foi uma descoberta notável durante a nossa pesquisa. Enquanto trabalhava com ágar Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) e utilizando uma estirpe de *V. ichthyoentri* gentilmente cedida pelo Dr. Manuel Manchado (Puerto de Santa Maria, Cádiz, Espanha), que foi obtida na água dos tanques de solha senegalesa (Solea senegalensis), a nossa equipa fez uma observação extraordinária. Em vez

de observar uma única cor uniforme na placa de ágar TCBS, identificaram dois fenótipos distintos. Um deles apresentava fermentação de sacarose, enquanto o outro não apresentava. Essa diferença na utilização de sacarose tornou-se a força motriz por trás da nossa jornada científica para descobrir as razões genômicas e moleculares subjacentes para essa divergência intrigante, a fim de compreender o seu comportamento. O processo começou com a separação cuidadosa e o cultivo dessas estirpes, e posteriormente foram submetidas a sequenciação para obter uma compreensão mais profunda dos fatores que contribuem para esses fenótipos distintos. O objetivo principal deste estudo é fornecer uma análise comparativa abrangente entre as duas estirpes de *V. ichthyoentri*, com foco particular em seus operões de sacarose em comparação com os de outras espécies de *Vibrio*. A abordagem investigativa combina ferramentas de bioinformática com ensaios microbiológicos, bioquímicos e moleculares para investigar os aspectos complexos da utilização de sacarose e a análise filogenética.

Ferramentas de bioinformática foram inicialmente utilizadas para anotar com precisão os genomas das duas estirpes de *V.ichthyoenteri*. Utilizando a ferramenta RAST, foi realizada uma comparação genômica com a estirpe de referência *V. ichthyoenteri* (ATCC 700023), revelando uma significativa semelhança na maioria das regiões genômicas. Essa semelhança reforça a sua classificação como *V. ichthyoenteri*; no entanto, as diferenças observadas no conteúdo genômico as distinguem como novas estirpes dentro desta espécie. A anotação da ferramenta RAST também revelou diferenças no número de genes entre as duas estirpes, sendo que *V. ichthyoenteri* 1 possui 4711 genes, enquanto *V. ichthyoenteri* 2 possui 4643 genes, e as principais diferenças residem nos genes relacionados a processos funcionais/metabólicos. De forma significativa, a pesquisa concentrou-se no operão de sacarose, que normalmente consiste em um conjunto de quatro genes responsáveis pela utilização da sacarose. Esses genes incluem o gene para o sistema de transporte de sacarose (scrA), um gene que codifica a sacarase (scrB), o gene da fructoquinase (scrK) e o gene codificador do regulador (scrR). No caso de *V. ichthyoenteri* 1 (estirpe não fermentadora de sacarose), a investigação revelou a presença de dois operões de sacarose (1A e 1B), enquanto *V. ichthyoenteri* 2 (estirpe fermentadora de sacarose) possuía apenas um operão. Esse achado destaca uma diferença notável na organização genômica dos operões de sacarose dessas duas estirpes. A análise filogenética revelou que *V. ichthyoenteri* 1A se assemelha mais a outros Vibrios, enquanto *V. ichthyoenteri* 1B e 2 são idênticos, sugerindo trajetórias evolutivas divergentes entre eles. Além disso, ao examinar a similaridade de sequência dos genes do operão de sacarose usando o recurso de relatório estatístico do programa GeneDoc, foram observadas diferenças significativas: *V. ichthyoenteri* 1A exibiu um grau maior de similaridade com genes ortólogos encontrados em *V. kanaloae* em comparação com *V. ichthyoenteri* 2. Isso implica uma semelhança genética entre *V. ichthyoenteri* 1A e V. kanaloae no que diz respeito aos genes do operão de sacarose. Por outro lado, os genes de *V. ichthyoenteri* 1B foram encontrados como idênticos (100% de similaridade) aos presentes em *V. ichthyoenteri* 2, fornecendo apoio adicional para as conclusões da árvore filogenética. A análise de ligação gênica também indicou a presença de clusters de genes relacionados ao Plasmídeo F nas proximidades do locus do operão de sacarose de *V. ichthyoenteri* 1A, sugerindo a possibilidade de eventos de transferência horizontal de genes (HGT).

Posteriormente, foram realizados ensaios microbiológicos para explorar a cinética de crescimento das duas estirpes de *V. ichthyoenteri* na presença e ausência de sacarose. Para este propósito, foram preparadas três composições de meios diferentes: a) TSB/1% NaCl, b) Peptona/1% NaCl e c) Peptona/1% NaCl/1% de Sacarose. Ambas as estirpes foram colocadas em microplacas com diferentes meios e, utilizando um leitor de microplacas a uma temperatura de 24-26°C, a densidade ótica (DO) a 600nm foi registrada a cada hora por aproximadamente 24 horas. Ambas as estirpes apresentaram crescimento muito semelhante no meio TSB/1% NaCl. Por outro lado, a presença de sacarose no meio de cultura contendo apenas Peptona/1% NaCl parece não influenciar o crescimento de ambas as bactérias, mas a sacarose provavelmente é metabolizada por *V. ichthyoenteri* 2, conforme evidenciado por mudanças na cor nos ensaios com TCBS e vermelho fenol. Foram realizados ensaios de atividade de esterases e proteases em condições específicas, incluindo TSA/1% NaCl a um pH de 7,3 ± 0,2 e uma temperatura de 22°C. Esses ensaios foram conduzidos tanto na presença quanto na ausência de 1% de sacarose, e o período de incubação durou 48 horas. Os resultados revelaram que a presença de sacarose levou a uma redução na atividade de enzimas que podem estar relacionadas à virulência. Finalmente, técnicas de biologia molecular, particularmente a análise de PCR do gene de utilização da sacarose (scrB), foram empregadas para investigar as mudanças na expressão gênica em resposta à presença ou ausência de sacarose. Os resultados indicaram que esse gene é expresso em ambas as estirpes, independentemente da presença de sacarose, com aumento da expressão ao longo do tempo.

No entanto, o mecanismo preciso responsável pela diferença na utilização de sacarose entre as duas estirpes ainda precisa ser explorado mais a fundo.

Palavras-chave: *Vibrio ichthyoenteri*, vibriose, aquicultura, metabolismo da sacarose, operão de sacarose

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List of Abbreviations

- **ARGs –** Antibiotic resistance genes
- **bp –** Base pair
- **CDC –** Centers for Disease Control and Prevention
- **CT –** Cholera toxin
- **CO_2** – Carbon dioxide
- **DNA** – Deoxyribonucleic Acid
- **DT –** Duplication time
- **EI –** Enzyme I
- **EI –** Enzymatic Index
- **FAO** – Food and Agriculture Organization of the United Nations
- **F-Plasmid** – Fertility factor Plasmid
- **gDNA** – Genomic DNA
- **HGT –** Horizontal Gene Transfer
- **HPr –** Histidine-containing protein
- **IHNV** – Infectious hematopoietic necrosis virus
- **Inc F-Plasmid –** Incompatible F-plasmid
- **IPNV –** Infectious pancreatic necrosis virus
- **ML**– Maximum Likelihood
- **NaCl** – Sodium chloride
- **NCBI –** National Center for Biotechnology Information
- **OD –** Optical density
- **OMPs** – Outer membrane proteins

- **PhyML –** Phylogeny Maximum Likelihood
- **PTS –** phosphoenolpyruvate (PEP)-dependent sugar phosphotransferase system
- **PCR** – Polymerase Chain Reaction
- **qPCR** – Quantitative Polymerase Chain Reaction
- **QS –** Quorum sensing
- **RAS –** Recirculating Aquaculture System
- **RAST –** Rapid Annotation using Subsystem Technology
- **RNA** – Ribonucleic Acid
- **rRNA** – Ribosomal RNA
- **TAE –** Tris-acetate-EDTA
- **TCBS** – Thiosulfate Citrate Bile and Sucrose
- **tRNA** – Total RNA
- **TSA** – Tryptic Soy Agar
- **TSB** – Tryptic Soy Broth
- **VNN** – Viral nervous necrosis

1- Introduction

1.1 The importance of Aquaculture in the global food supply

Aquatic food products are one of the most traded food commodities in the world and their consumption has increased nearly five times in the last 60 years (1). Aquaculture, or the farming of aquatic animals and plants, play a crucial role in the global food chain and is one of the fastest growing food sectors with an average annual growth rate of 5.8% (2). This industry contributes with 49.2% (60 million tons) (Figure 1) of the global production of aquatic animals and 52% of the fish produced for human consumption, evaluated in USD 250 billion (1). It is expected that aquaculture will remain crucial for the world food supply in the next decades. According to the United Nations, an increase in the world population of almost 50 % to 9.7 billion is expected between 2000 to 2050 (3). It is also expected that aquaculture production will double to meet the 58% increase in human fish consumption until 2050 while the wild fisheries will keep declining (Figure 2)(4). Fish and shellfish are the most produced aquaculture products and, in 2020, farmed finfish production reached 57.5 million tons (USD 146.1 billion) and shellfish production (mostly bivalve mollusks) reached 17.7 million tons (USD 29.8 billion)(1). Aquaculture also constitutes an alternative to wild capture fisheries, that have been declining in the last decades. Well-managed aquaculture systems can provide a reliable, sustainable, controlled, and high-quality source of marine food products without pressuring the natural systems and assuring the quality of the products (5). Another key benefit of aquaculture is the fact that this system helps to reduce pressure on wild stocks as overfishing and unsustainable fishing practices contributed to the depletion of many fish and shellfish stock populations in their natural environment(6).

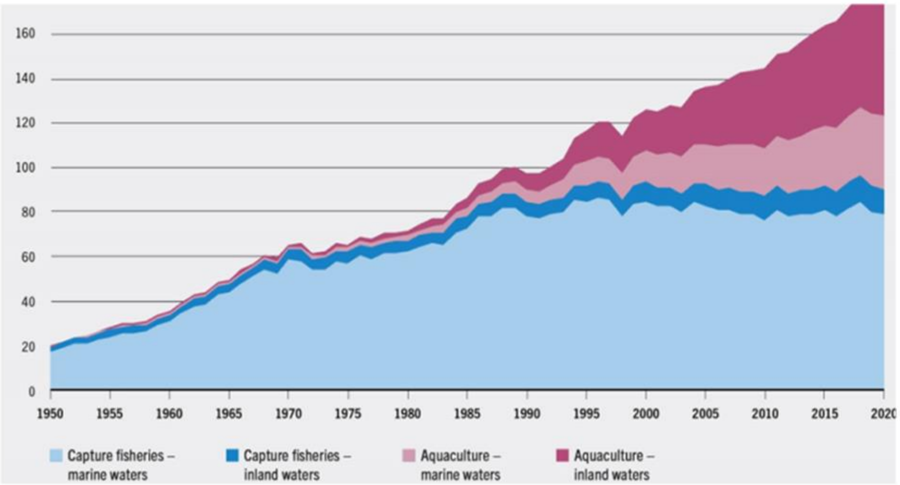


Figure 1 World Capture Fisheries and Aquaculture Production (1950-2020). This figure represents amount in Million tons of the total capture and aquaculture production from 1950 to 2020. This image was obtained from(1).

In the next decades the demand for animal-based protein will undergo a two-fold increase by 2050 but the expansion of the existent land-based food production systems (which provide most of the available food in the world) will be unsustainable as it is already reaching the limits of the planet sustainability (7,8). Land-based systems occupy 50 % of habitable land, are highly dependent of fossil fuels and use 70 % of the available freshwater resources (9,10). On the other hand, aquaculture systems do not require such natural resources and can be expanded as the aquatic ecosystems (wetlands, rivers, lakes, and coastal estuaries) represent 75 % of the planet surface but remain largely unexplored. Despite the advantages of aquaculture, only a relatively small percentage of the aquatic resources are used for aquaculture production and the number of farmed species is very low (11,12).

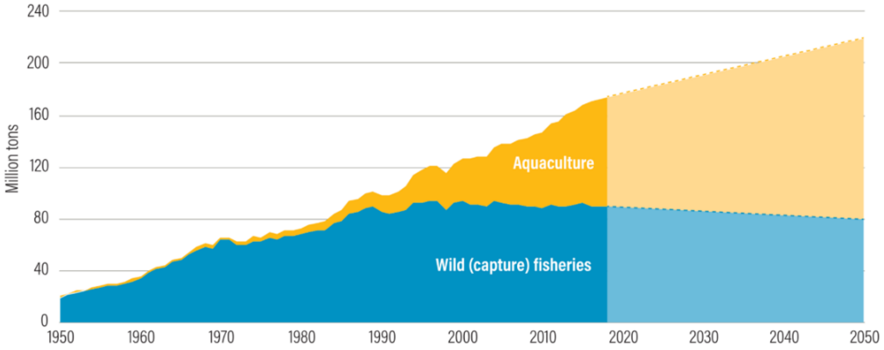


Figure 2 Historical and Projected Global Seafood Production. Historical data, 1950–2016: FAO (13) and FAO (8). Projections to 2050: Calculated at WRI; assumes 10 percent reduction in wild fish catch from 2010 levels by 2050, linear growth of aquaculture production of 2 Mt per year between 2010 and 2050. Source (1)

Aquaculture also provides economic benefits to communities as it creates employment opportunities and contributes to the development of local economies (14,15). In fact, the worldwide economic worth of cultivated fish might ascend from $120 billion in 2010 to $308 billion in 2050. Concurrently, the number of individuals reliant on aquaculture for their livelihoods could elevate from 100 million in 2010 to 176 million by the middle of the century (16).

1.2 Bottlenecks of aquaculture expansion

The expansion of the aquaculture industry is hindered by several tailbacks that need to be addressed to achieve sustainable production levels of high-quality protein seafoods. One of the major bottlenecks fish and shellfish aquaculture production are microbial disease outbreaks caused by marine pathogens, that are becoming more frequent due to the increase of intensive aquaculture production systems to increase food production (2,17,18). Marine pathogens, such as bacteria, viruses, fungi, and parasites, represent a significant threat to the health and productivity of farmed aquatic animals. Disease outbreaks caused by marine pathogens result in high mortality rates, reduced growth rates, and consequently decreased production yields and are responsible for billions of dollars losses annually (19,20). Infectious microbial diseases have an overall global impact of more than 6 billion dollars per year and in specific aquaculture sectors (such as shrimp), losses may exceed 40 % of the global production with emergent diseases threatening to collapse several productions across Asia (21,22). Furthermore, the widespread application of antibiotics and other chemicals to control the growth and proliferation of these pathogens results in environmental contamination and the emergence of antibiotic-resistant bacterial strains which pose additional risks to the aquaculture productivity and to human health (23,24). Moreover, aquaculture conditions modify the composition and function of the microbial communities, resulting in aquaculture-specific niches (25).There are several pathogens that accumulate in the marine organisms and are posteriorly transmitted to humans when contaminated fish and seafoods are consumed raw or undercooked and are then responsible for seafood-borne diseases outbreaks that poses a serious public health problem. Microbial pathogens produce several virulence factors that help the colonization and invasion of the host tissues, leading to tissue damage (26). These virulence factors are located either on the surface of the bacteria or are secreted to the surrounding environment such as membrane and secretory proteins, a polysaccharide capsule, components of the outer membrane, siderophores, and biofilm formation proteins (17,18,20). Understanding these virulence factors and pathogen physiology to create management strategies play a critical step towards the development of preventions and mitigation strategies to ensure sustainability and productivity of this industry (27). Effective strategies for the prevention and control of disease outbreaks include improved biosecurity measures, vaccination programs, and the development of alternative disease control strategies to improve the host defense mechanism to reduce the reliance on antibiotics and other chemicals (28,29). Below is a brief description of some of the main groups of pathogenic microbes that have an impact on aquaculture.

1.3 Marine pathogens

1.3.1 Pathogenic bacteria

Bacteria are accountable for a spectrum of infectious illnesses in a wide array of marine organisms, encompassing fish, invertebrates, and corals, and they can cause serious infections in both farmed and wild marine organisms (30–33). Aquaculture environments provide the optimal conditions for fish or shellfish farming but also provide the optimal conditions for bacterial growth and proliferation. Since marine bacteria can survive in the water column and do not exclusively depend on host for their survival, their presence is very common and aquaculture where they cause several infection diseases (34). Some of the most common pathogens found in marine aquaculture systems include bacteria of the *Vibrio* genus, which are abundant in the marine environment and part of the marine microbiota. However, a few species of this genus are primary or opportunistic pathogens that can infect marine organisms, causing vibriosis. The presence of *Vibrio* species in aquaculture has been linked to disease outbreaks in oysters, shrimp, and finfish farms (35). In addition, other bacterial genus such as *Pseudomonas*, *Aeromonas*, and *Edwardsiella* have also been identified as most commonly pathogens of fish and implicated in disease outbreaks (36). This thesis is about Vibrios and a detailed explanation about this bacteria genus is now provided.

1.3.2 Virus

Viruses can pose a significant threat to aquaculture. Different types of viruses have been recognized as challenges in this field, including infectious hematopoietic necrosis virus (IHNV) and infectious pancreatic necrosis virus (IPNV). They mainly affect fish like rainbow trout and salmon, causing significant mortality (37,38). Moreover, other viruses such as betanodaviruses are responsible for viral nervous necrosis (VNN) in fish, which impacts the nervous systems of fish and causes remarkable fatality rates (39). Furthermore, herpesviruses infections result in a range of clinical signs such as tiredness, reduced appetite, and death. Viruses that accumulate in marine organisms can also be transmitted to humans when contaminated seafood is consumed. Some examples are norovirus and hepatitis A, that can accumulate in shellfish and be responsible for 83.7 % and 12.8 % of human foodborne outbreaks, respectively (40).

1.3.3 Other marine pathogens

In marine aquaculture systems, infections caused by fungi and parasites are also very problematic. For instance, genera like *Fusarium* and *Aspergillus* represent common fungal agents affecting marine life, including various aquatic organisms such as Indian major carps, salmonids, rainbow trout (41). An impressive case involves *Exophiala salmonis*, a fungal pathogen responsible for mortality rates of up to 50 percent in Atlantic salmon smolts and post-smolts (42)(Figure 3). Also, *Ichthyophonus* is a significant pathogen that plays a role in the decrease of Atlantic herring populations (20). Apart from fungi, parasitic infections pose a substantial concern, with Trematodes and protozoans serving as prime examples of parasitic agents. Within the genus *Perkinsus*, a well-known protozoan gives rise to perkinsosis, exerting a significant influence on molluscan aquaculture (43).

Figure 3*:* *Exophiala* Fungus-Induced Black Pigmentation in Cod. Whole fillet of cod on ice, showing characteristic black pigmentation of infected musculature by *Exophiala* sp. (fungus producing melanin pigments). Target hosts: Cod (*Gadus morhua*), marine-reared salmonids, and several other marine fish species. Source (20)

1.4 Bacteria of the *Vibrio* genus

1.4.1 General characteristics

The *Vibrio* genus englobes marine rod-shaped bacteria that belong to the *Gammaproteobacteria* class (the most diverse class of Gram-negative bacteria). They belong to the *Vibrionaceae* family along with seven other genera that include *Aliivibrio*, *Catenococcus*, *Enterovibrio, Grimontia, Listonella, Photobacterium, Salinivibrio*. *Vibrio* species are characterized by having two circular chromosomes (Chromosome I and II) with distinct sizes, along with the presence of numerous cryptic plasmids and they are highly heterogeneous and polyphyletic bacteria clade (25,44–46). The most diverse clades are Harveyi, Splendidus, and Cholerae which include pathogens of marine organisms or humans (47,48)(Figure 5). Chromosome I is the largest with a size of approximately 3 (Mb) and its sequence is highly conserved within the *Vibrio* genus, containing essential bacterial genes involved in basic cell maintenance and survival such as replication, transcription, and translation. On the other hand, Chromosome II is the smallest and exhibits variable sizes across different *Vibrio* species and it carries genes related to phenotype traits (47,49,50). *Vibrio* is the genus with the highest number of identified species among the other genera of the same family and accounts with 147 species and 4 subspecies of which most are naturally present in the marine ecosystems and only few are pathogenic. This genus is also found as part of the gastrointestinal tract of numerous aquatic species or in symbiotic relationships with other marine organisms (44,46,51). Vibrios are naturally found in aquatic environments, particularly warm, slightly salty (brackish) waters typically within 20°C and 37°C (47). Physiologically, *Vibrio* species are notable for their rapid growth rates and high ability to swiftly adapt to favorable environmental conditions such as higher temperatures, increased salinity, and elevated dissolved oxygen levels (49,52). Vibrios exhibit distinctive biochemical and physiological characteristics and are recognized for their capacity to produce oxidase enzymes, generate indole, and utilize citrate. Additionally, they can reduce nitrate to nitrite. They play an important role in the marine environment as they are involved in the mineralization of organic matter and in the recycling of organic materials due to their ability to break down a diverse range of substances, including carbohydrates, lipids, and proteins. Moreover, they possess the capability to degrade complex polymers such as gelatin, collagen, starch, chitin, alginate, lignin, and hydrocarbons. This active degradation process significantly contributes to the recycling of carbon and nitrogen within aquatic environments. Furthermore, Vibrios can produce polyunsaturated fatty acids, which are essential compounds for organisms in the aquatic food web that lack the capacity to synthesize them independently. Interestingly, *Vibrio* bacteria themselves can also serve as a food source for flagellates, further enhancing the recycling of organic matter within aquatic ecosystems (46,47,53)(Figure 4).

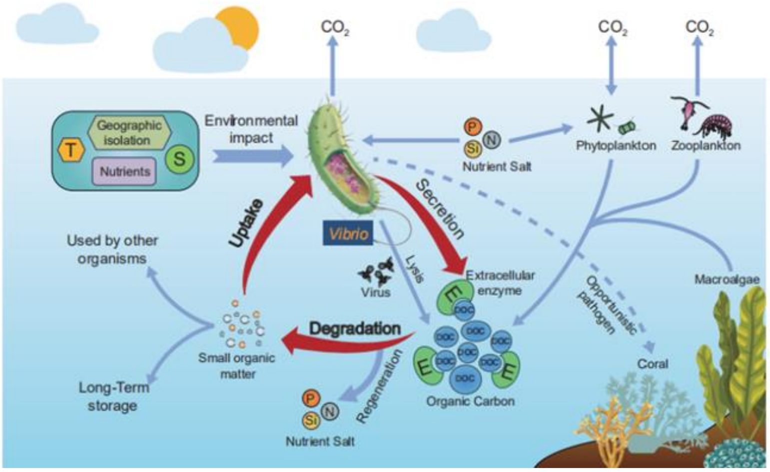


Figure 4. The Contribution of *Vibrio* Species to the marine carbon cycle. The impact of *Vibrio* species on this cycle is influenced by various environmental factors, which in turn affect the secretion of extracellular enzymes. These enzymes are responsible for breaking down organic carbon compounds, making them available for utilization by other marine organisms. T- temperature; S- salinity; P- phosphate; N- nitrogen salt; Si- silicate; E- extracellular enzyme; POC- particulate organic carbon; DOC- dissolved organic carbon. Source(53).

1.4.2. Pathogenic *Vibrio* species

While Vibrios undeniably fulfill an ecologically significant role in aquatic environments, a few species are key pathogens in the marine environment and represent a major threat to aquatic organism and even human health (Figure 5). Their high abundance, substantial biomass, and capacity for independent growth and highly adaptability to different environments make them a primary source of infectious diseases in the aquatic environment. Four species *V. harveyi, V. vulnificus, V. alginolyticus*, and *V. parahaemolyticus* are associated with the most common vibriosis outbreaks that cause significant economic losses in the aquaculture industry. Additionally, 12 are recognized human pathogens, being of particular concern the bacteria *V. alginolyticus, V. parahaemolyticus, and V. vulnificus* (54). Human infections caused by *Vibrio* species generally occur through the consumption of contaminated seafood, the intake of contaminated water, direct contact of skin injuries with contaminated waters, and person- to-person transmission (less common) (25). According to the estimates from the Centers for Disease Control and Prevention (CDC), vibriosis are responsible for approximately 80,000 illnesses and 100 deaths annually in the United States, with 65% of the cases related to the consumption of contaminated seafood (55). Human diseases caused by pathogenic *Vibrio* can be categorized into two primary groups: *cholera* and non-cholera infections. *V. cholerae* is identified as the primary etiological agent responsible for cholera, a severe diarrheal illness. In contrast, non-cholera *Vibrio* species, such as *V. parahaemolyticus* and *V. vulnificus*, are accountable for a spectrum of infections and clinical manifestations of vibriosis are wound, ear infection and gastroenteritis (25).

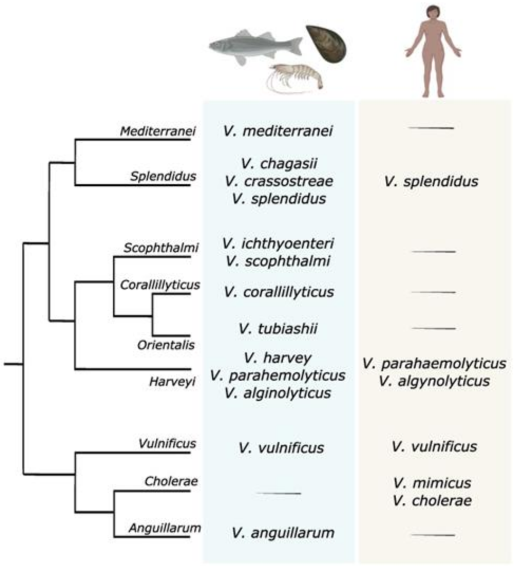


Figure 5. Major marine and clinical *Vibrio* pathogens. Dendrogram represents the evolutionary relationship between the different clades and was designed based on a phylogenetic analysis using Multi-locus Sequence Alignment of 5 genes (*gapA*, *ftszZ*, *mreB*, *topA* and *gyrB*) (56). Pathogenic bacteria clades are represented, and examples of the main species are given. The image was drawn using BioRender and Inkscape. Source(57).

1.4.3 The burden of vibriosis in Aquaculture

In aquaculture, vibriosis affecting reared finfish, shellfish, and shrimp lead to significant economic losses (51). The outcome and severity of the disease rely on several factors such as the host species and immune system, the bacterial strain and virulence factors, the concentration of the pathogenic agent, the duration of the infection, and the prevailing environmental conditions. Several *Vibrio* species are recognized as opportunistic pathogens (58,59) despite being present in high concentrations in the core microbiota of the tank water and marine organisms, but also present in live food and *Vibrio* infections are likely to occur. Among the most frequently identified species in aquaculture are *V. anguillarum, V. ordalii, V. vulnificus*, *V. alginolyticus, V. parahaemolyticus, V. harveyi* and *V. tubiashii*. Although *V. cholerae* is not typically considered a primary fish pathogen, it has also been isolated from both freshwater and marine fish. These animals serve as a broad reservoir of *V. cholerae* strains that have the potential to cause infections in humans. However, it was recently reported that a non-O1, non-O139 *V. cholerae* serotype (EMM1) capable of inducing significant mortality in the freshwater species (35). This suggests that *V. cholerae* strains, beyond the typical human pathogens, should also be regarded as relevant aquatic pathogens. In aquaculture vibriosis can be triggered by sudden changes in water temperature, poor water quality and fluctuations in water salinity (18,60). In fish the primary routes of transmission are the skin, gills, and gastrointestinal tract and the symptoms of vibriosis usually include lethargy, tissue and appendage necrosis, reduced rates of growth and metamorphosis, developmental malformations, muscle opacity, and tissue myelinization (61). Disease outbreaks caused by this type of bacterial pathogens cause multi-million-dollar losses (Table 1) and the economic burden is estimated at 1.05 to 9.58 billion US$ per year. Moreover, the incidence of outbreaks is predicted to increase so understanding *Vibrio* behavior and monitoring their presence in the marine environment and in marine organisms is essential to develop preventive strategies and mitigate the occurrence disease outbreaks contributing to better marine health and more effective aquaculture management practices (17).

Table 1. Examples of reported vibriosis outbreaks in fish and shellfish farms. The pathogen is indicated, along with the main symptoms, and cost to industry. Source: (57)

Country	**Year**	**Pathogenic agent**	**Losses and impact**
Taiwan	2001	*V. alginolyticus*	Lethargy, dark skin and ascites in the peritoneal cavity, and damaged eyes of juvenile cobia (Rachycentron canadum)
Tunisia	2010	*V. parahaemolyticus*	High mortality of European Seabass (Dicenthracus labrax)
India	2003	*V. parahaemolyticus* *V. alginolyticus* *V. anguillarum* *V. vulnificus*	Slow growth and mortality of shrimp (Penaeus monodon)
China	2011-2013	*V. mimicus*	High mortality rate (80%–100%) of freshwater catfish
Egypt	2014	*V. anguillarum* *V. alginolyticus* *V. ordalii* *V. harveyi*	50% mortality of Seabass and Seabream
China	1990-1992	*V. fluvialis*	>US$ 120M annual losses
Indonesia	1991	*Luminescent Vibrio*	>US$ 100 M loss at shrimp hatcheries

1.5. Challenges in controlling Vibriosis

1.5.1 Environmental parameters

Climate change, emerging from the release of greenhouse gases like carbon dioxide, methane, and nitrous oxide since the Industrial Revolution, presents an additional factor that influences the presence, proliferation and virulence of marine (20,62,63). Climate models project a temperature rise of 2.5–10 °C in the next century, causing water quality disturbances that include temperature shifts (rising or falling), hypoxia, CO_2 buildup (lowering pH), precipitation (altering salinity), and changes in storm and cyclone frequency and intensity (20). These disruptions have immediate impacts on the equilibrium between hosts and marine pathogens, particularly the susceptibility of the host to the pathogen, the bacterial growth, the abundance and virulence of the pathogens, and the emergence of novel pathogenic bacteria (64)(Figure 6). Growth kinetics of bacteria are notably affected by variations in water temperature and chemical composition and infections caused by *Vibrio* species exhibit a noticeable seasonal pattern. In warmer waters the proliferation of certain *Vibrio* species, such as *V. parahaemolyticus,* can be notably enhanced. Some examples of the impact of increased seawater temperatures triggering disease outbreaks also include: vibriosis in marine salmonids caused by *V. ordalii* (65) *V. harveyi* infections in shellfish European abalone (66) and *V. shiloi*-induced coral bleaching in corals (67). Salinity levels also play a crucial role in environments with both low and high salinity; however, its impact is more difficult to assess. While the adhesion and growth of *V. cholerae O139* can be adversely affected by lower salinity levels, *V. parahaemolyticus* tends to exhibit a positive correlation with growth. Furthermore, the pH range of the water can also exert significant effects. Members of the *Vibrio* genus can grow in a broad pH range, and this can lead to both positive and negative effects depending on the species and in several marine pathogens pH decrease was detrimental for growth and virulence (68,69).

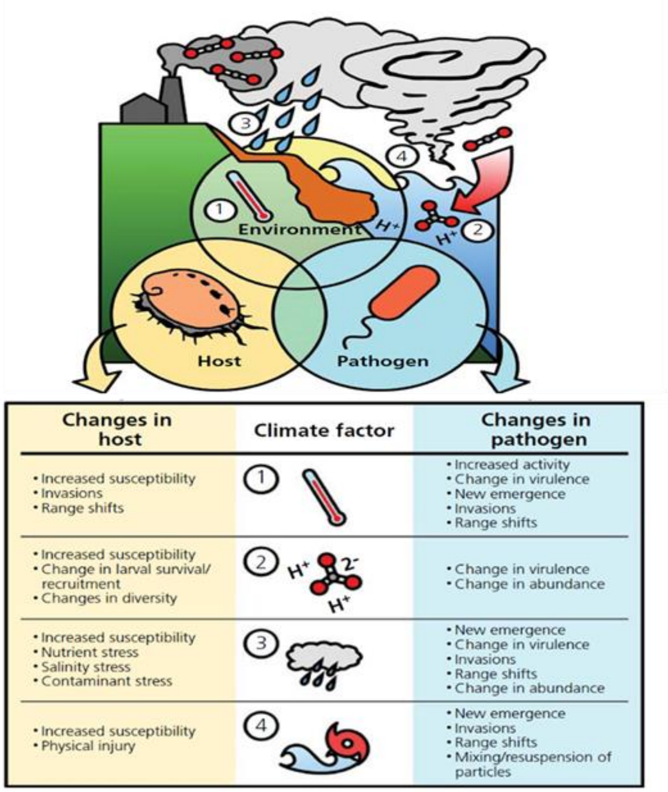


Figure 5 The effects of climate change on marine interactions between hosts, pathogens, and the environment. Global environmental shifts are causing alterations in the physical oceanic conditions, encompassing (1) temperature fluctuations, (2) variations in CO_2 levels resulting in pH changes, (3) shifts in precipitation leading to salinity variations, (4) and increase exposure to storms and cyclones. Consequently, these factors are causing a reconfiguration of the equilibrium between hosts, pathogens, and the environment. Source (20).

1.5.2 Emergence of novel *Vibrio* strains

Vibrio species have two chromosomes that are subject to frequent recombination and intense horizontal gene transfer (HGT) events that lead to the frequent emergence of novel strains with modified behaviors and virulence (70). This genetic flexibility is explained by their natural competence that enables the integration of external DNA into their genome through diverse HGT mechanisms. This plasticity allows *Vibrio* species, like *cholerae* (one of the most studied), known for its adaptability, to effectively respond to a wide range of environmental stresses, including antimicrobial challenges (71). Several *Vibrio* species have acquired resistance to common antibiotics used in aquaculture because there is a rapid transfer of antibiotic resistance genes (ARGs) among members of this genus (72). Moreover, molecular analyses comparing pre-pandemic and pandemic strains of *V. parahaemolyticus* have shown a potential link between the emergence of pandemic strains and recombination and HGT events (73).

A significant factor influencing *Vibrio* behavior is chitin, a fibrous polysaccharide found freely in the marine environment, and in the exoskeletons of crustaceans and fish scales. High concentrations of chitin trigger an increase in competence and DNA uptake capability in *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus* by providing a platform for *Vibrio* species to attach and engage in the exchange of genetic information (25,74). Three primary routes of horizontal gene transfer (HGT) are recognized within the bacteria, including transduction, transformation, and conjugation (Figure 7). Transduction is the process in which a virus (bacteriophage- virus that infects bacteria) transfers genetic material to another bacterium. Bacteriophages can encapsulate sections of the host's DNA in their capsid and when host cell lysis occurs due to environmental triggers, they inject this DNA into a new host that can undergo recombination with the cell's chromosomal DNA. Transformation is a process where a bacterial cell's genetic makeup changes due to the direct intake, integration, and utilization of external DNA from closely related bacteria. This state is triggered by factors like low nutrient availability or high cell density. Conjugation is a process of horizontal gene transfer in which the genetic material of one bacterium (donor), often in the form of a plasmid, is transferred to another bacterium (recipient) through direct physical contact. This transfer occurs through a structure called a pilus, which connects the two cells and is the most common mechanism used to the spread of traits such as antibiotic resistance and virulence factors (20,71,75).

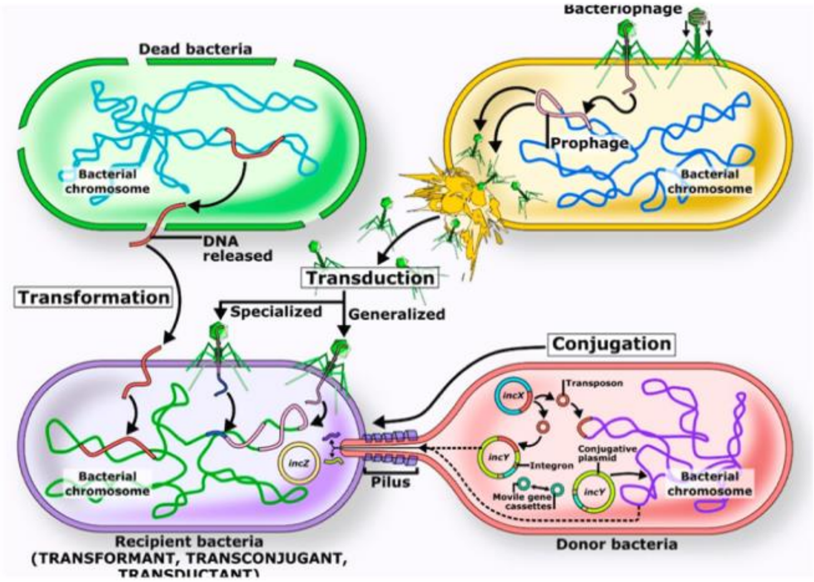


Figure 7 Bacterial Evolution through DNA Exchange Mechanisms (HGT). Genetic diversity and adaptation in bacteria are driven by the transfer of DNA. This figure illustrates the three main mechanisms of DNA transfer described in prokaryotic organisms: transformation, transduction, and conjugation. These mechanisms play a crucial role in endowing bacteria with genes that confer resistance to antibiotics, metals, pathogenicity, symbiosis, and metabolism of new substrates. Through these processes, bacteria acquire and share genetic information, contributing to their ability to evolve and adapt in changing environments. Source (75)

The National Center for Biotechnology Information (NCBI) database contains over 18,000 *Vibrio* genomes, with around 2,050 corresponding to different pathogenic strains. It is estimated that more than 30% of the *Vibrio* species present in the marine environment, including novel pathogenic strains, are transferred from commercial aquaculture systems due to poorly managed conditions (18,76). Recently our research group focused on the characterization of potential novel *Vibrio* species that were isolated from the water and tissues from diseased Senegalese sole (*Solea senegalensis*) from aquaculture. Using classical microbiological and molecular tools, seven putative novel strains, including pathogenic and non-pathogenic species, were characterized (47). Within the isolates were two putative novel isolates of *V. ichthyoenteri* as revealed by biochemical and molecular phylogenetic characterization (Figure 8 and 9). Phylogenetic analysis revealed that the two *V. ichthyoenteri* strains cluster in the same branch as the reference strain available in the NCBI database (AFWF01000053) within the Scophthalmi clade but they are slightly different (Figure 8) and biochemical characterization of the two novel strains revealed that they have distinct behaviors regarding the metabolization of sucrose and their morphological characteristics on Thiosulfate citrate Bile-Salt Sucrose (TCBS) agar plates (Figure 9).

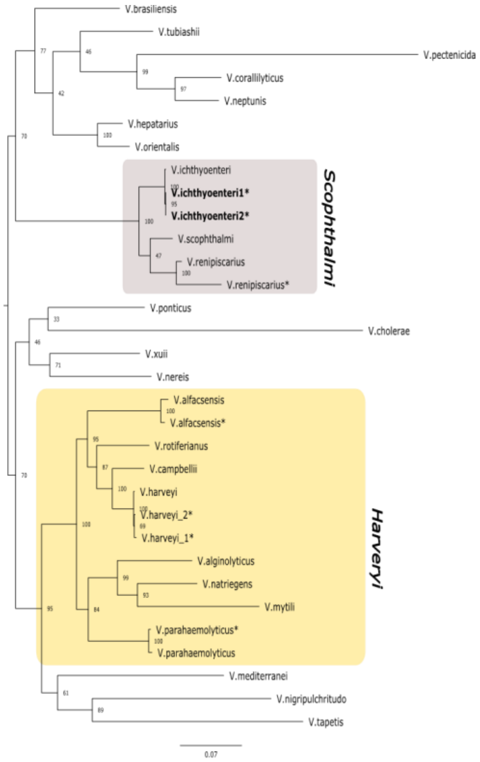


Figure 8 Phylogenetic tree of *Vibrio* spp. Tree was constructed using five concatenated housekeeping genes: *ftsZ* (cell division protein FtsZ), *gapA* (glyceraldehyde 3-phosphate dehydrogenase), *gyrB* (DNA gyrase subunit B), *mreB* (rod shape-determining protein MreB), and *topA* (DNA topoisomerase I). The housekeeping genes revealed the two *V. ichthyoenteri* strains cluster in the same branch as the reference strain available in the NCBI database (AFWF01000053) which is part of the Scophthalmi clade. Source (47)

TCBS is a unique culture medium designed for the specific isolation and cultivation of *Vibrio* species and provides a means of distinguishing different *Vibrio* species based on their ability to utilize sucrose. The isolates capable of fermenting sucrose produce acid byproducts that lower the pH of the medium which causes the modification of colony color from the original green to a yellow shade (77). One *V. ichthyoenteri* strain showed yellow colonies while the other showed green colonies (Figure 9). This differentiation in behavior suggests that there might be significant variations in their metabolic pathways or the genetic content of the sucrose operon as it plays a central role in the utilization of sucrose by bacteria. The two strains were designated *V. ichthyoenteri* 1 (non-metabolizer) and *V. ichthyoenteri* 2 (metabolizer) and are the focus of this study which aims to understand the genetic basis for their distinct sucrose fermenting phenotypes by looking at the sucrose metabolizing system (sucrose operon) and its functions.

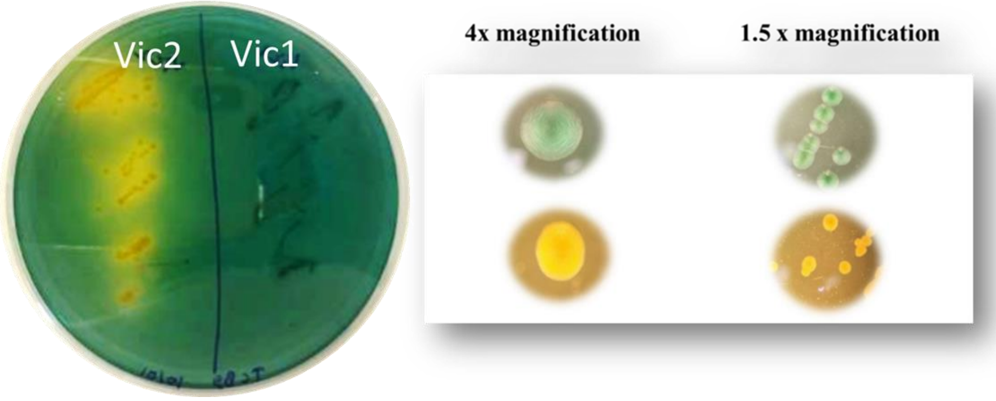


Figure 9. The two *V. ichthyoenteri* isolates on TCBS agar plates after 48h incubation at 22 ºC. *V. ichthyoenteri* 1 (non-metabolizer of sucrose) and *V. ichthyoenteri* 2 (metabolizer of sucrose) left panel. Digital photographs of representative colonies on TCBS plates at 22 °C. Note that Vic1 is green and Vic2 is yellow. Images were obtained using two different magnifications (4 x and 1.5 x). Source: (28)

1.5.2.1 The bacterium *V. ichthyoenteri*

The bacterium *V. ichthyoenteri* was formally named by Ishimaru et al. and this pathogen was initially isolated in the 1970s in Japan from the gut of diseased Japanese flounder larvae but was not studied in detail until late 80s (78). These bacteria, such as other Vibrios, is a gram-negative, facultatively anaerobic that has a straight or slightly curved rod shape and is motile through a single polar flagellum (Figure 10). It has an extensive geographic distribution from Asia to Europe and America, but currently no different strains have been reported and only one reference genome is available in the public database and until today there are no reports of other diseases caused by this bacterium. It is estimated that the reference strain, *V. ichthyoenteri* F-2, has a genome with a total chromosome size of 4.6 Mb that includes a large chromosome of 3.2 Mb and a smaller chromosome of ~1.4 Mb (79). *V. ichthyoenteri* is a member of the Scophthalmi clade that contains only bacteria that are pathogenic to marine organisms but not to humans (unlike bacteria of the Harveyi clade). In fish, *V. ichthyoenteri* causes enteritis that mainly occurs in flatfish, especially in the larval fish of flounder and turbot and represents a severe disease in flounder hatcheries with mortality rates soaring to 90% (80)(81) (Figure 11). It usually causes intestinal necrosis in the early stages of olive flounder (*Paralichthys olivaceus*) when their stomach is not yet fully formed which makes them more vulnerable. In addition to flounder, other flatfish species, such as turbot, are also significantly affected by this pathogen (82). It was also reported that the species is a pathogen to Torafugu (Takifugu rubripes, a pufferfish) (83).

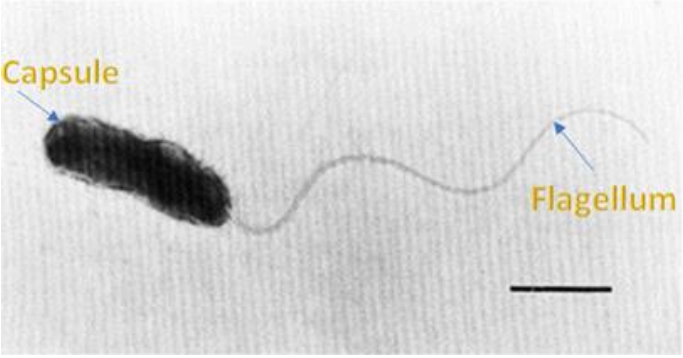


Figure 10. Electron micrograph of a negatively stained cell of *V. ichthyoenteri* F-2T. showing the single polar flagellum. Bar = 1 µm. Source (84)

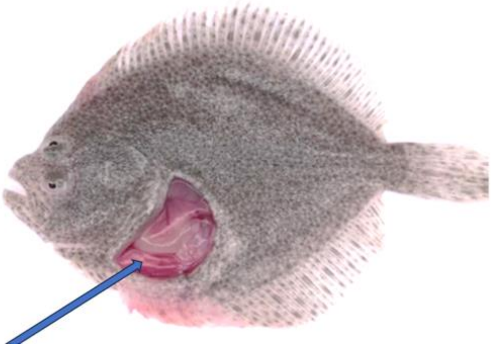


Figure 6 Turbot Infected with *V. scophthalmi* Strain VSc190401. Indicating similar symptoms of severe organ enteritis evident from the redness and inflamed characteristic of the intestine indicated with the blue arrow. This image was obtained from (85)

Compared with other *Vibrio* species still very little is known about *V. ichthyoenteri* behavior and infection. However, recent exploration of the Quorum Sensing (QS) system is shedding some light on the communication mechanisms utilized by this bacterium to govern essential functions like biofilm formation, virulence adaptation, and antimicrobial production (86). QS is a regulatory mechanism found in various bacterial species and involves the secretion of molecules that bind to receptors as cell density increases, triggering gene expression changes. *V. ichthyoenteri* possesses two QS systems like other *Vibrio* species. One system generates

AHL molecules like *V. harveyi's* AI-1 mechanism, while the other is aligned with *V. scophthalmi's* AI-2 system, as indicated by shared luxS genetic sequences. AI-2 activity and luxS-like genes play roles in various bacterial features. Interestingly, luxS in *V. ichthyoenteri* does not appear to regulate virulence gene expression (87). The typical approach to battle *V. ichthyoenteri* infections is the administration of antibiotics. However, the extensive use of antibiotics in aquaculture poses risks such as the emergence of antimicrobial resistance and environmental pollution (88–90). To address this issue, researchers have been focusing on developing an effective vaccine that target virulence protein factors. Outer membrane proteins (OMPs) have shown promise in this regard. These proteins are crucial for various aspects of bacterial infection, including adherence to host cells, iron transport, and resistance to being engulfed by immune cells (91). Among the OMPs studied, OmpT and OmpA have been identified as particularly promising vaccine candidates for combatting *V. ichthyoenteri* infections (82,91)

1.6 Sucrose metabolism in bacteria

Carbohydrates serve as vital nutrients for heterotrophic bacteria, offering them a carbon source for nourishment and growth. Sucrose is the most abundant carbohydrate in the marine environment and is characterized as a disaccharide formed by a glucose unit linked to a fructose unit through a glycosidic linkage (92). Many Eubacteria have developed specialized catalytic enzymes, such as sucrose-6-phosphate hydrolases and sucrose phosphorylases, enabling them to efficiently metabolize sucrose in a controlled manner. This metabolic pathway involves breaking down sucrose into simpler components (glucose and fructose) that can be utilized for energy production and growth (92,93). The extracellular enzymes involved are secreted to the environment to enable the degradation of the carbohydrate outside the cell and its posterior uptake and utilization. The enzymes involved in sucrose metabolization are encoded by the sucrose operon that is regulated in response to environmental signals and the presence of sucrose. Operons are common in prokaryotic organisms, where multiple genes with related functions are organized (clustered) and transcribed together under the control of a single promoter and regulatory elements (94).

1.6.1 The sucrose operon

The sucrose operon usually comprises a cluster of four genes that contains the gene for the sucrose transport system (*scrA*), a gene encoding hydrolase (*scrB*), the fructokinase gene (*scrK*), and the regulator encoding-gene (*scrR*) (Figure 12).

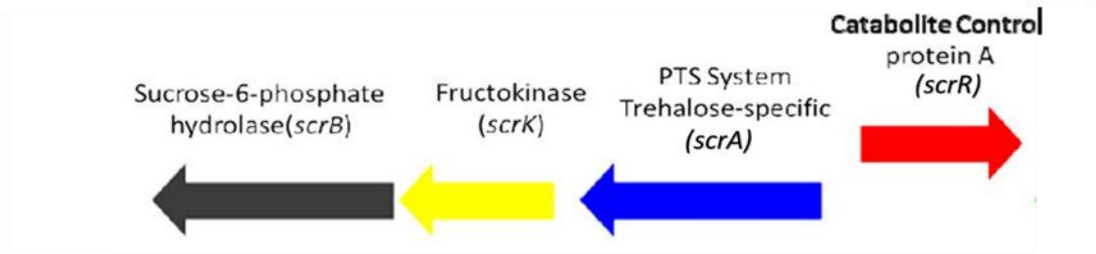


Figure 12. Classical sucrose operon organization found in bacteria genome. This operon comprises genes responsible for encoding key enzymes such as sucrose-6-phosphate hydrolase (*scrB*), fructokinase (*scrK*), and the PTS System (*scrA*) Additionally, the operon includes the sucrose repressor gene (*scrR*), which plays a pivotal role in the regulatory control of this operon and the gene orientation is the opposite of others. Adapted from:(48).

1.6.1.1 Sucrose transport systems

Bacterial cells utilize membrane transport proteins to facilitate the entry of carbohydrates. These proteins often consist of transmembrane helical segments and may involve one or more protein components (92,95). One of the major mechanisms for the transport of sugars in bacteria (Figure 13) is by the phosphoenolpyruvate (PEP)-dependent sugar phosphotransferase system (PTS) and is encoded by the *scrA* gene. The bacterial PTS serves as a mechanism for both the uptake and phosphorylation of sucrose but also plays a role in signal transduction. This system encompasses two essential phosphotransferase proteins, namely EI (Enzyme I) and HPr (Histidine-containing protein), along with a variable number of sugar-specific enzyme II complexes (IIA, IIB, IIC) that differ based on the bacterial species. Within this system, EI and HPr facilitate the transfer of phosphoryl groups from PEP to the IIA units. Subsequently, the IIA and IIB units sequentially transfer phosphate groups to the sugar molecule. As a final step, the IIC unit translocate the phosphorylated sugar across the membrane. There are alternative mechanisms for bacterial sucrose transport to the intracellular region in addition to PTS. These non-PTS sugar permeases facilitate sucrose accumulation without chemical modification (92,95). Non-phosphotransferase-dependent sucrose metabolic system (non-PTS system) involves facilitated diffusion of sucrose via a sucrose-H+ symport system.

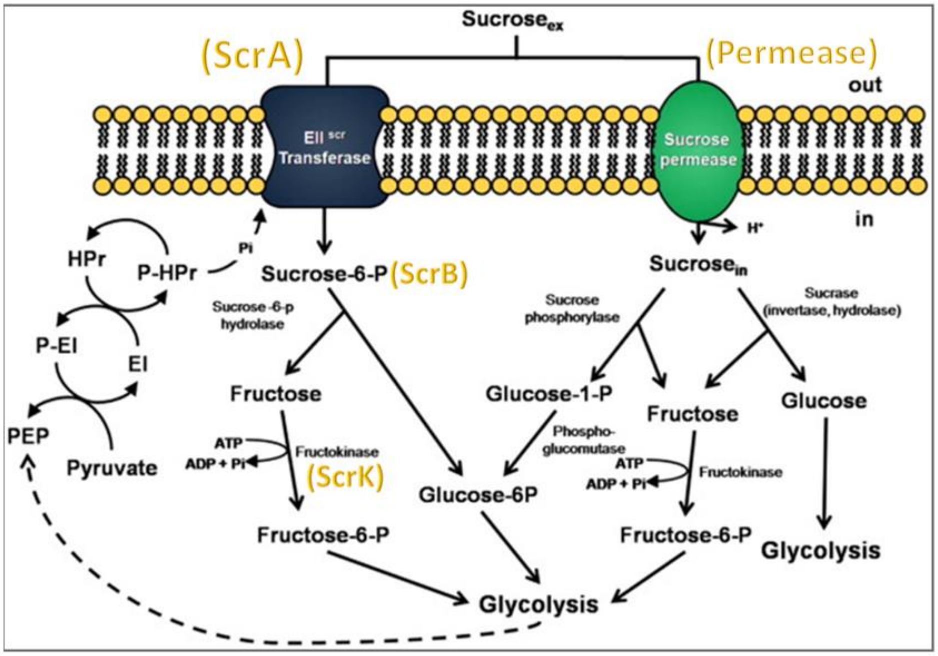


Figure 13 Schematic representation of the 2 sucrose-utilization systems (ScrA and permease). Representing two different pathways by which sucrose enters the cell and the processes leading to the glycolytic cycle. Source:(96).

1.6.1.2 Sucrose-6-phosphate hydrolase (*scrB*) and fructokinase (*scrK*) genes

Whether sucrose is taken up via the PTS system (ScrA) or permeases, its processing within bacterial cells follows distinct pathways (Figure 13). When sucrose enters through the PTS System, it undergoes hydrolysis, resulting in the production of intracellular glucose-6-phosphate and fructose. This hydrolysis is achieved through the action of sucrose hydrolases (ScrB enzymes), which play a pivotal role in cleaving sucrose-6-phosphate into its constituent glucose and fructose components (Figure 13). Conversely, in cases where sucrose is transported via permeases, it enters the bacterial cell unmodified. Then hydrolysis of sucrose into its basic glucose and fructose components occurs, and after that fructose proceeds to enter subsequent metabolic pathways and is converted into fructose-6-phosphate by the fructokinase enzyme, that is encoded by *scrK* gene. The resulting fructose-6-

phosphate, and the glucose-6-phosphate enter the glycolytic pathway (Glycolysis) for energy production (92)(Figure 13).

1.6.1.3 Sucrose operon repressor (scrR)

Regulation of the sucrose operon is dependent on the sucrose availability in the medium and the scarcity of the preferred carbon sources (glucose) (95). The sucrose operon is an inducible operon and, as a result, the promoter is freed in the presence of sucrose and absence of glucose, allowing mRNA production to proceed. This mRNA production triggers the transcription of the genes within the operon and subsequently the metabolization of sucrose (Figure 14). Regulation of the sucrose operon depends on the binding of the repressor protein (ScrR) to the operator region in the DNA, which blocks the assembly of the RNA polymerase and inhibits the transcription. The repressor present in the sucrose operon is encoded by the *scrR* gene and is a member of the LacI-GalR family, which is involved in the regulation of the transcription of a wide range of processes (97). These proteins are characterized by having a monomeric structure with both DNA-binding and regulatory domains and homodimer formation is required for binding to operator DNA (97). Studies using *V. alginolyticus* identified putative binding sites for the Scr repressor protein in the sucrose operon which are found in the intergenic region between the *scrA* and *scrR* genes (98).

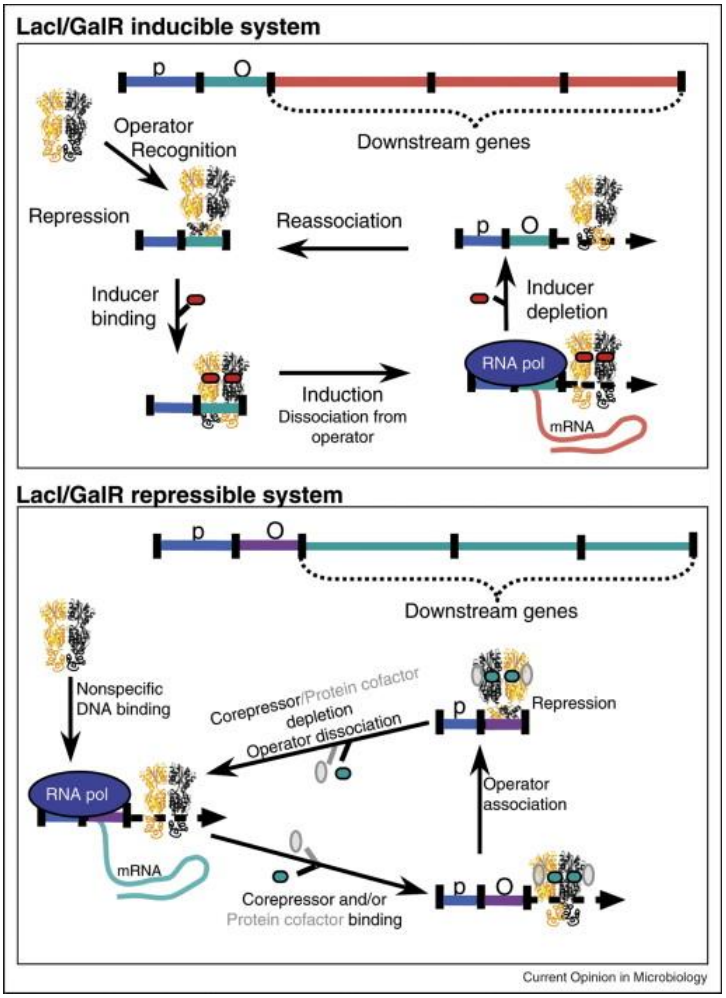


Figure 14. The inducible and repressible systems of LacI/GalR protein cycles. In the Inducible system the inducer (depicted as red) binds to the repressor protein make conformational changes which reduce its affinity for the operator resulting in dissociation of operator and the start of transcription. In the repressible system the presence of its cognate co-repressor ligand (teal) and/or cofactor protein (gray) trigger a conformational change in the repressor resulting in higher affinity for its target DNA site, which in turn decreases transcription. Source(97)

1.6.2 The sucrose operon in *Vibrio* species

Most of the *Vibrio* species can ferment sucrose. However, the human pathogen *V. parahaemolyticus* is considered as a non-sucrose fermenter bacterium because its genome has lost all the genes that are part of the sucrose operon(99). Within the *Vibrio* genus the bacterium *V. alginolyticus* is the most studied to understand the process of sucrose uptake and breakdown. and its pathway aligns with a common carbohydrate utilization pathway observed in many other bacteria. The sucrose operon is composed of four genes for sugar-specific PTS uptake (*scrA*), fructokinase (*scrK*), sucrose-6-phosphate hydrolase (*scrB*), and a regulatory gene (*scrR*) (Figure 15). Most *Vibrio* species also have the porin gene (*scrY*) next to the operon that is not part of the operon but is localized upstream the *scrR* gene and is a transmembrane protein facilitating sucrose uptake during conditions of growth limitation (100).

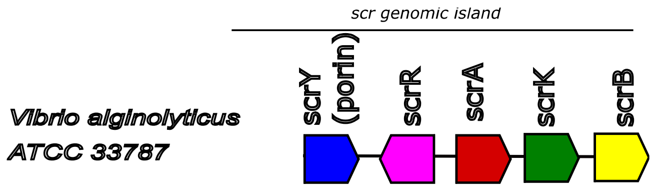


Figure 15. Genomic organization of the sucrose operon in *V. alginolyticus*. The colored arrows represent the genes and arrow end points the direction they are in the genome. The porin genes (*scrY*) is not part of the classical sucrose-operon but is present in many *Vibrio* spp. genomes. The structure of the *V. alginolyticus* sucrose operon was obtained from(101).

However, due to gene transfer mechanisms, some traditional non-sucrose fermenters can acquire the capacity to metabolize sucrose or distinct sucrose fermentation capabilities. The bacterium *V. parahaemolyticus* has been the most studied in this regard (gene transfer mechanisms) because is a human pathogen and recently 17 sucrose-positive *V. parahaemolyticus* strains were identified. Such evidence results from intense gene transference between different bacteria, which emphasizes the significance of horizontal gene transfer in obtaining novel metabolic capabilities and phenotypic traits that may also influence their virulence (101).

In one study, the expression of virulence-associated genes in *V. cholerae* associated with the presence of various carbon sources was studied (102). It was specifically examined if the accumulation of cholera toxin (CT), a major virulence determinant, in culture supernatants and the results demonstrated that CT production was higher in the presence of glucose, whereas the presence of lactate led to lower production levels (Figure 16). Moreover, when sucrose was present there was a reduction of CT production to less than approximately 75% (reduced almost 25%) compared to the levels observed under glucose culture and this highly suggested that sucrose utilization has a direct impact on bacteria virulence (102). It has also been described that sucrose metabolization in *V. cholerae VCA0653* is related with the impediment of biofilm formation (103). These findings emphasize the complex relationship between the utilization of carbohydrates and the expression of virulence factors on pathogenic bacterial species.

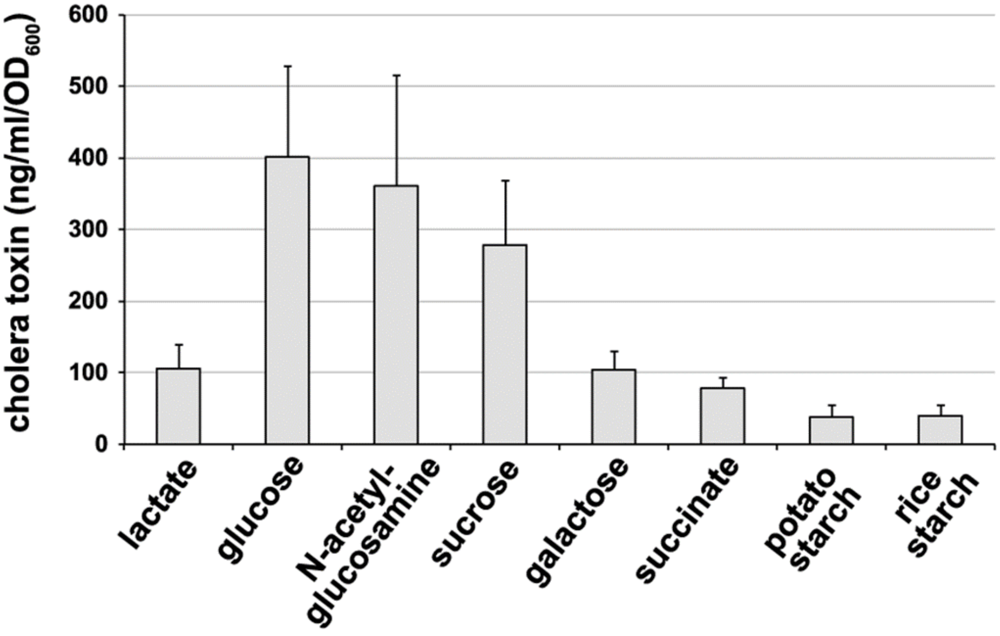


Figure 7 Quantification of cholera toxin upon growth on diverse carbon sources. The result showed that in sucrose case cholera toxin decreased to less than approximately 75% compared to the levels observed under glucose. Source:(102)

1.7 Objective

Bacteria of the *Vibrio* genus are one of the most successful microbes in the marine environment and pathogenic species represent a major risk to aquaculture and human health. The incidence of vibriosis outbreaks in aquaculture and seafood-borne diseases is increasing following the crescent consumption of seafood products, the consequent intensification of the aquaculture industry and the impact of climate change on host-pathogen-environment interactions. Moreover, aquaculture systems are a major source of emerging novel *Vibrio* strains, potentially pathogenic, due to their optimal conditions for bacterial growth and genetic transference. The characterization of *Vibrio* species in the marine environment and emerging novel strains are thus essential to understand their behavior and pathogenicity and their potential risk for aquaculture production or human health. The aim of this study was to elucidate on the genetic and functional differences of sucrose utilization of two novel *Vibrio* strains by providing a comparative study using bioinformatic, microbiological, biochemical, and molecular assays:

a) Bioinformatics is used to annotate the genomes of the two *V. ichthyoenteri* strains, characterize sucrose operon genes and compare them to other *Vibrio* species.
b) Microbiological assays will be used to assess the impact of the presence and absence of sucrose on the growth kinetics between the two *V. ichthyoenteri* strains.
c) Biochemical assays will be used to assess the effect of sucrose on the enzymatic activity (proteolytic and hydrolytic activities) associated with their potential virulence.
d) Molecular biology is used to characterize the changes in gene expression of the sucrose utilization gene (scrB) in the presence and absence of sucrose.

2- Material and Methods

2.1. Isolation and maintenance of the *V. ichthyoenteri* isolates

The two isolates of *V. ichthyoenteri (V. ichthyoenteri1 and V. ichthyoenteri2*) used in this study were generously provided by Dr. Manuel Manchado (Puerto de Santa Maria, Cadiz, Spain) and were obtained from tank water of Senegalese sole (*Solea senegalensis*) cultivated in Recirculating Aquaculture System (RAS) indoor facilities. The isolates were maintained on Tryptic Soy Agar (TSA) supplemented with 1% NaCl (TSA/1% NaCl) (pH 7.3 ± 0.2) at 22 °C and -liquid cultures of the bacterial isolates were performed in Tryptic Soy Broth (TSB) supplemented with 1% NaCl (TSB/1% NaCl) with a pH of 7.3 ± 0.2 and incubated overnight (~16h) at 22 °C with shaking at 150 rpm.

2.2 Genome annotations

2.2.1. Genome annotation

The genomes of the two novel *V. ichthyoenteri* strains were sequenced by a company using Illumina sequencers (HiSeq/NovaSeq) and DNA for sequencing was prepared by the group members. The sequences were preliminarily annotated by the company. The raw reads were used and gene prediction, identification of protein-coding genes and assignment of gene functions through their categorization into subsystems based on specific biological process or structural complex was performed with the Rapid Annotation using Subsystem Technology (RAST) tool available from (https://rast.nmpdr.org/). The RAST tool provides high-quality genome annotations for prokaryotes and classifies protein coding genes in different subsystems which are defined as functional roles that together implement a specific biological processor structural complex. The genome raw reads of the two bacteria were uploaded in FASTA format and analyzed using the Domain Bacteria and Genetic Code for 11 (Archaea, most Bacteria, most Virii, some Mitochondria) option according to the classic RAST method. Data was downloaded and analyzed in the SEED-Viewer's comparative environment.

2.2.2 Identification of sucrose operon

The genes related to sucrose utilization in *V. ichthyoenteri* such as sucrose porin *(scrY),* catabolite control proteins *(scrR)*, PTS System sucrose/trehalose-specific *(scrA)*, fructokinase *(scrK),* and sucrose-6-phosphate hydrolase *(scrB)* were text-searched from the annotated genome files provided by the company and the nucleotide and predicted protein sequences were retrieved for further analysis. The structure of the operon was obtained by determining the position and orientation of each gene in the sucrose operon cluster provided.

2.3 Comparative analysis

The sucrose operon genes were also identified using a similar strategy in other *Vibrio* genomes available in house for *V. parahaemolyticus* (Vpa)*, V. alfacsensis* (Val)*, and V. renipiscarius* (Vre), and two isolates of *V. harveyi* (Vha1 and Vha2). Homologue genes were also searched in the reference genomes for other *Vibrio* species (*V. aestuarianus*, *V. vulnificus*, *V. furnissii*, *V.diazotrophicus*, *V. navarrensis*, *V. alginolyticus*, *V. chagasii*, *V. cholerae*, *V. splendidus,* and *V. crassostreae*) and reference bacteria genomes for *Escherichia coli* UMN026, *Erwinia amylovora, Streptococcus mutans, Klebsiella pneumoniae,* and *Salmonella spp*. available from the NCBI database (https://www.ncbi.nlm.nih.gov). Gene positions were verified using graphical annotations available and gene orientation and structure were obtained.

2.4 Multiple sequence alignments and phylogenetic analysis

The nucleotide and deduced protein sequences of the sucrose operon genes and five housekeeping genes of *V. ichthyoenteri* and other *Vibrio* species were extracted and compared using multiple sequence alignments and phylogenetic trees. All the multiple sequence alignments were performed using the AliView tool according to the MUSCLE (alignment software) and the output file was saved as “Phylip" format for further analysis in PhyML (Phylogeny Maximum Likelihood), a software tool specifically designed for phylogenetic analysis using the Maximum Likelihood (ML) method (http://www.atgc-montpellier.fr/). All ML analysis was performed using 100 bootstrap replicates and LG was the substitution model used for the proteins automatically selected using the BIC (Bayesian Information Criterion). Trees obtained using the nucleotide sequences were performed using the GTR matrix. The tree outputs were visualized in FigTree(104) and edited in Inkscape.

To compare the evolution of the sucrose operon genes in relation to *Vibrio* species, multi-locus sequence analysis was performed by concatenating the sequences of the sucrose operon in different species and strains and compare that to the species evolution tree. For the trees nucleotide sequences were used as they yield more phylogenetic information than protein. The evolutionary trees were performed concatenating the sequences of the four sucrose operon genes and the species tree with the concatenated sequences of five housekeeping genes: *ftsZ* (cell division protein FtsZ), *gapA* (glyceraldehyde 3-phosphate dehydrogenase), *gyrB* (DNA gyrase subunit B), *mreB* (rod shape-determining protein MreB), and *topA* (DNA topoisomerase I) retrieved from a total of 19 *Vibrio* species (56). These selected housekeeping genes were previously shown to be highly effective in distinguishing between different taxa (such as species or closely related groups) of the *Vibrionaceae* family(105). MLSA and trees were performed as described above but the model for the nucleotide trees was the GTR automatically selected using the BIC (Bayesian Information Criterion) and 100 bootstrap replicates were used. The percentage of sequence similarity of the deduced proteins of the sucrose operon genes were performed using GeneDoc program.

2.5 Gene neighboring analysis

To better understand the evolution of the sucrose operon the genes that flank the sucrose operon locus were identified and compared between the two strains. For that, the annotated files of the deduced proteins were used to identify the neighboring genes in the two species and their sequences were retrieved and searched against the NCBI database to characterize their identity as most were unknown according to the initial annotation. Several genes that were previously classified as hypothetical proteins, their identity was obtained and genome maps for each operon were manually constructed based on their position in the genome contigs and strand. The neighboring sequences of the *V. ichthyoenteri* 1 sucrose operon on contigs 36 and 70 were text searched in the genome of *V. ichthyoenteri* 2 and sequence homologues were also procured in the reference genome for this species available from NCBI

(GCA_000222605.2, *V. ichthyoenteri* (taxid:142461)) using blastp tool. Similarly, the *V. ichthyoenteri* 2 was searched in *V. ichthyoenteri* 1 and in the reference species genomes.

2.6 Bacteria growth curves

The isolates were cultured on TSA/1% NaCl and incubated at 22 °C for 48 hours. For the overnight culture, a loopful of each bacterium from the TSA/1% NaCl plate was transferred into 5 mL of TSB/1% NaCl and cultures were subsequently incubated at 22 °C for approximately 18 hours at 150 rpm. For the construction of the growth curves two different mediums were selected, TSB and Peptone. TSB was selected because is a rich general medium where *V. ichthyoenteri* strains grow optimally, and Peptone media was used because is a minimal nutrient medium that allows the manipulation of the carbohydrate source. The *Vibrio* strains were cultured in the two different media in the presence or absence of sucrose to determine the impact of sucrose on their growth kinetics. Three different media compositions were prepared: a) TSB/1% NaCl, b) Peptone/1% NaCl, and c) Peptone/1% NaCl/1% Sucrose. The sucrose was previously filtered through a 0.22 µm filter and added to the media after autoclaving to guarantee that the disaccharide properties of sucrose remained unaffected. Twenty microliters of the overnight culture were inoculated in 180 µL of each media composition in a sterile 96-well microplate (Sarstedt, Germany) with a lid. Three technical replicates (n=3 wells) for each condition were assigned for each *V. ichthyoenteri* strain and specific media. The microplate was placed in a Microplate reader (on a Synergy Neo2 Hybrid Multi-Mode Multiplate Reader (Biotech, USA)) at 24-26°C and the optical density (OD) at 600_{nm} were recorded every hour for ~24 h. This essay was repeated three times. The specific growth rate (µ) was calculated from the average slope of the exponential growth phase of each isolate on a semilogarithmic scale, and the duplication time (DT) was obtained by dividing ln (2) by value of µ (the specific growth rate) obtained from the slope on the semilogarithmic plot.

2.7 Extracellular enzyme activity

Enzymes secreted by bacteria play a crucial role in their virulence by enabling them to break down various substrates and colonize host organisms. To assess the enzymatic activity of *V. ichthyoenteri* and evaluate the impact of sucrose on the enzyme activity, esterase and protease activity assays were conducted in the presence and absence of 1% sucrose in the culture medium. The bacterium *V. harveyi* was used as a positive control. The assays were conducted on TSA/1% NaCl and pH of 7.3 ± 0.2 using 1% Tween 20 and 1% gelatin to measure Esterase and Protease activity, respectively. Esterase enzymes hydrolyze the ester bonds present in Tween 20, and the extent of ester bond hydrolysis serves as a measure of esterase activity. On the other hand, proteases produced by the *Vibrio* isolates hydrolyze the gelatin, resulting in visible clear zones of hydrolysis around the colonies. The colonies used for both Esterase and Protease activity assays were obtained from the most recent cultures grown on TSA plates to ensure their viability and active growth state. Enzymatic Index (EI) was calculated as described by (Florencio et al. 2012(106) .

2.8 Genomic DNA extraction

Genomic DNA (gDNA) was isolated to optimize the species-specific primers developed for the gene expression studies. The gDNA was obtained using a modified version of the GES method initially described by Pitcher et al, 1989 (107). The isolates were incubated in TSB/1% NaCl (pH 7.3 ± 0.2) at 22 °C for approximately 16 ± 2 hours at 150 rpm and the cultures were centrifuged at 13,000 rpm for 10 minutes to obtain the bacterial pellet. This pellet was resuspended in 100 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) and then 500 µL of GES solution (consisting of 60 g Guanidium Thiocyanate, 20 ml of 0.5 M EDTA pH 8, 5 mL of 10% Sarkosyl, and distilled water to achieve a final volume of 100 mL) and 6 µL of RNase (10 mg/mL) (Sigma, Spain) were added. The reaction was incubated at room temperature for 10 minutes and 250 µL of 10M Ammonium Acetate was added to precipitate the proteins. After gentle inversion and incubation on ice for 10 minutes, 500 µL of chloroform/isoamyl alcohol (24:1) solution was introduced to eliminate proteins and the reaction was centrifuged at 13,000 rpm for 10 minutes at 4°C.The supernatant, which formed a two-phase solution, was collected in a new Eppendorf tube. To precipitate the gDNA, 400 µL of cold isopropanol was added to the supernatant, followed by vortex and centrifugation at 13,000 rpm for 5 minutes at 4°C. The resulting gDNA pellet was washed three times with 400 µL of cold 70% ethanol, each time centrifuging at 13,000 rpm for 5 minutes at 4°C. The pellet was then air-dried for 10 minutes to allow ethanol evaporation, after which it was resuspended in 50 µL of water purified using a MilliQ system (Millipore, USA). The quality and concentration of the extracted gDNA were assessed using a NanoDrop (ThermoFisher Scientific, USA), and the integrity of the gDNA was evaluated by running 5 µL of the DNA sample (initial concentration of 50 ng/µL) on a 1% agarose gel with 1x Tris-Acetate-EDTA (TAE: 40 mM Tris-base, 20 mM Acetic acid, 1 mM EDTA) electrophoresis.

2.9 Primers design

Specific primers to amplify a fragment of the *scrB* gene (239 bp) were manually designed by colleagues based on the sucrose operon gene nucleotide sequence. The primers are designed to amplify the *scrB* gene in cluster 34 of *V. ichthyoenteri 1* and the orthologue gene in cluster 33 of *V. ichthyoenteri 2*, which are identical in sequences. The sequence of the primers used for *scrB* and *16S* rRNA genes are available in Table 2.

Table 2: List of the primers used in the PCR reactions

Primer	Sequence 5’ to 3’	Accession number	Annealing temperature (ºC)
16SF	*CCCAGATGGGATTAGCTTGT*	JQ904752.1	50
16SR	*TCTGGACCGTGTCTCAGTTC*		
ScrBF	*ATTGGAGATTAAAAGGCGAGC*		60
ScrBR	*CTCATCCCAATGAGTGACTGT*		

2.10 RNA extraction and cDNA synthesis

2.10.1 Total RNA extraction

To characterize the sucrose gene expression, total RNA (tRNA) was extracted from the bacterial pellets collected from different culture time points (0, 4, 8, and 24 h) grown on 50 ml of Peptone/1% NaCl with and without sucrose. Five ml of each culture were collected and centrifuged 5000 rpm for 5 min to separate the cell pellet from the culture media. RNA was extracted using the extraction kit ENZA, total RNA Kit I (OMEGA- bio-tek, USA) by resuspending the pellet in 500 mL of lysis buffer and resuspended by agitation for 2 minutes to promote cell lysis. Samples were incubated on ice for 10 minutes and centrifuged at 12,000 rpm for 10 minutes at 4°C, enabling the separation of cellular debris, and the supernatant containing the RNA was carefully transferred to new tubes. Next, 550 ml of cold 70% ethanol was added to the supernatant, and the mixture was agitated for 20 seconds. The samples were poured into the column tubes and centrifuged at 13500 rpm for 1 minute to bind the RNA to the column. Next, 500 mL of wash buffer 1 is added to the column, incubated at room temperature for 2 minutes, and centrifuged for 1 minute at 13500 rpm. The eluate was discarded, and the process repeated with 500 mL of wash buffer 2, followed by centrifugation at 13500 rpm for 1 minute. The wash step with wash buffer 2 was repeated once again, and the column was centrifuged for 3 minutes at a maximum speed of 13,500 rpm to dry from any remaining liquid. To elute the purified tRNA, 40 mL of nuclease-free water was added to the column and incubated for 1 minute, followed by centrifugation for 2 minutes at 13,500 rpm. The eluate containing the RNA was collected and placed on ice.

2.10.2 Genomic DNA treatment

To eliminate any potential traces of genomic DNA that may interfere with the amplification reactions a DNAse treatment was performed. To tRNA eluate, 4 mL of 10 X Reaction buffer and 0.5 mL of DNase enzyme (from the PRIMER DESIGN DNase Kit, UK) were added and the samples incubated at 30°C for 20 minutes to allow DNase digestion of residual genomic

DNA. After the DNase treatment, the sample was incubated at 55°C for 5 minutes to inactivate the DNase enzyme and ensure complete DNA digestion.

2.10.3 cDNA synthesis

Five hundred ng of tRNA were used to produce cDNA. To the adequate volume of tRNA, 10 ng of random hexamer primer (RH, Jena Bioscience, Germany) and water were added to a final volume of 13,5 mL The mixture was carefully mixed to ensure proper homogeneity and was incubated in a PCR machine at 65 °C for 5 minutes followed by cooling at 4 °C for 5 minutes to denature the RNA and prevent hairpin formation. The cDNA synthesis reaction continued with the addition of the following reagents to the mixture: 100 U of reverse transcriptase enzyme, 2 μM of dNTPs, 8 U of RNase inhibitor, and 4 μL of 5 x Reverse transcriptase buffer (ThermoScientific, USA) reaching the final volume of 20 μL. The cDNA synthesis reaction was carried out in a PCR machine with the following temperature and time settings: 10 minutes at 22 °C for initial denaturation and priming, followed by 60 minutes at 42 °C for reverse transcription to synthesize the complementary DNA strand. Finally, the reaction was incubated at 72 °C for 5 minutes to ensure completion of the reverse transcription process.

2.11 Polymerase chain reaction (PCR)

PCR on genomic DNA was performed to optimize the primer condition for the posterior assessment of the gene expression. The reaction was prepared with 2 μL of gDNA (50 ng/μL); 2 μL 10x DreamTaq Buffer (Thermofisher, USA); 0.5 μL of 20 mM dNTPs (Thermofisher, USA); 0.5 μL of each primer (10 mM); 0.125 μL of DreamTaq polymerase 5 U (Thermofisher, USA) for a final volume of 20 μL. The thermocycle conditions consisted of 95 °C for 3 minutes, 35x (95 °C for 20 seconds (sec), X °C for 20 sec (X = 56ºC, 60ºC, 64ºC), 72 °C for 20 sec), and a final extension at 72 °C for 5 minutes. The success of each reaction was analyzed on 1,5% agarose/1x TAE gel electrophoresis and was observed using the Image Lab Software GelDoc XR+ (Bio-Rad, USA) to detect the amplicons of expected size. The amplicons were sequenced using the Sanger method at the CCMAR facilities using the automated sequencer Applied Biosystems 3130xl Genetic Analyzer (Applied

Biosystems, USA) and the BigDyeTerminatorv3.1 kit. Their identity was confirmed by analyzing their nucleotide sequences against the NCBI database (https://blast.ncbi.nlm.nih.gov) using the BLASTn algorithm to retrieve similar nucleotide sequences. For the expression studies reactions PCR on cDNA was prepared with 1 µL of cDNA of each sample; 1.5 µL 10x DreamTaq Buffer (Thermofisher, USA); 0.3 µL of 20 mM dNTPs (Thermofisher, USA); 0.3 µL of each primer (initial concentration of 10 mM); 0.06 µL of DreamTaq polymerase 5 U (Thermofisher, USA) for a final volume of 15 µL. To assess the quality of the cDNA produced, a PCR on 16S rRNA unit was performed. The thermocycle conditions consisted of 95 °C for 3 minutes, 35x (95 °C for 20 seconds (sec), 60 °C for 20 sec, 72 °C for 20 sec), and a final extension at 72 °C for 5 minutes. For the *scrB* genes the conditions used were the same, but the number of cycles was 25 to avoid saturation and allow a better characterization of the target gene expression profile. The success of each reaction was analyzed on 1,5% agarose/1x TAE gel electrophoresis and was observed using the Image Lab Software GelDoc XR+ (Bio-Rad, USA) to detect the amplicons of expected size. Quantification of the intensity of the amplified products for expression comparison was calculated using Quantity Tool available from the Software GelDoc XR+ to perform relative comparative analysis.

2.12. Statistical analysis

All graphics were performed in Microsoft excel, and statistical analysis was performed in the program GraphPad prism version 9 for windows. Triplicates were performed for each assay and t-test was used to evaluate for significant statistical differences between the enzymatic activity of the isolates.

3. Result and Discussion

3.1 Genome annotation of the two-novel *V. ichthyoenteri* strains

The genome sequences of both *V. ichthyoenteri* strains were annotated using the RAST tool and the overall assessment is shown in Table 3**.** Genome assembly of the putative *V. ichthyoenteri* 1 resulted in 206 contigs (contiguous stretch of DNA sequence that has been assembled from smaller overlapping DNA fragments) with a total length of 5,067,182 bp. Assembly of *V. ichthyoenteri* 2 resulted in 201 contigs with a total length of 5,009,200 bp, suggesting that *V. ichthyoenteri* 1 has a slightly bigger genome size. The average G+C content (which is a parameter related to genome organization and is typically associated with gene-rich regions) was identical between the two strains but the N50 (which is the shortest contig length that needs to be included along with the larger contigs for covering 50% of the genome) of *V. ichthyoenteri* 2 (117, 902 bp) was longer than *V. ichthyoenteri* 1 (110,821 bp).

Table 3 Overview of *V. ichthyoenteri1* and *V. chthyoenteri2* genome assemblies.

	V. ichthyoenteri 1	***V. ichthyoenteri 2***
Genome Length	5,067,182 bp	6,009,200 bp
G + C content	44.2%	44.2%
Predicted coding genes	4711	4643
N50	110,821bp	117,902 bp
Number of RNAs (ncRNAs)	131	140
Contigs	206	201

Functional subsystem annotation performed using the RAST tool predicted 4711 genes in *V. ichthyoenteri 1* and 4643 genes in *V. ichthyoenteri 2*. In *V. ichthyoenteri 1,* 51% of the genes (2429/ 4711) were not categorized to any specific biological process or structurally complex (subsystem). For *V. ichthyoenteri* 2 a similar proportion of genes were not attributed to a biological process or a structural complex (Figure 17). Total Number of Subsystems annotated for *V. ichthyoenteri1* was 517 and a close number was also predicted for *V. ichthyoenteri* 2 with 515. The main difference between the two strains resides in the number of genes in each functional/metabolic subsystem features which may reveal physiological differences between the two strains. In the subsystem related to Membrane Transport and the one related to Carbohydrates the *V. ichthyoenteri* 1 has more genes compared to *V. ichthyoenteri* 2, with 38 and 4 more genes, respectively. On the other hand, V. *ichthyoenteri* 2 had more genes related to DNA Metabolism and Fatty Acids (1 extra gene), Lipids and Isoprenoids (1 extra gene), and Amino Acids and Derivatives (3 extra genes) than *V. ichthyoenteri* 1. Sucrose utilization genes were identified under the carbohydrate subsystem (Figure 17), and it was observed that *V. ichthyoenteri 1* possessed 11 sucrose-utilization genes, while *V. ichthyoenteri 2* had 5 sucrose-utilization genes. This difference in gene content may explain their distinct behaviors regarding sucrose fermentation and to explore this hypothesis the sucrose operon was further characterized.

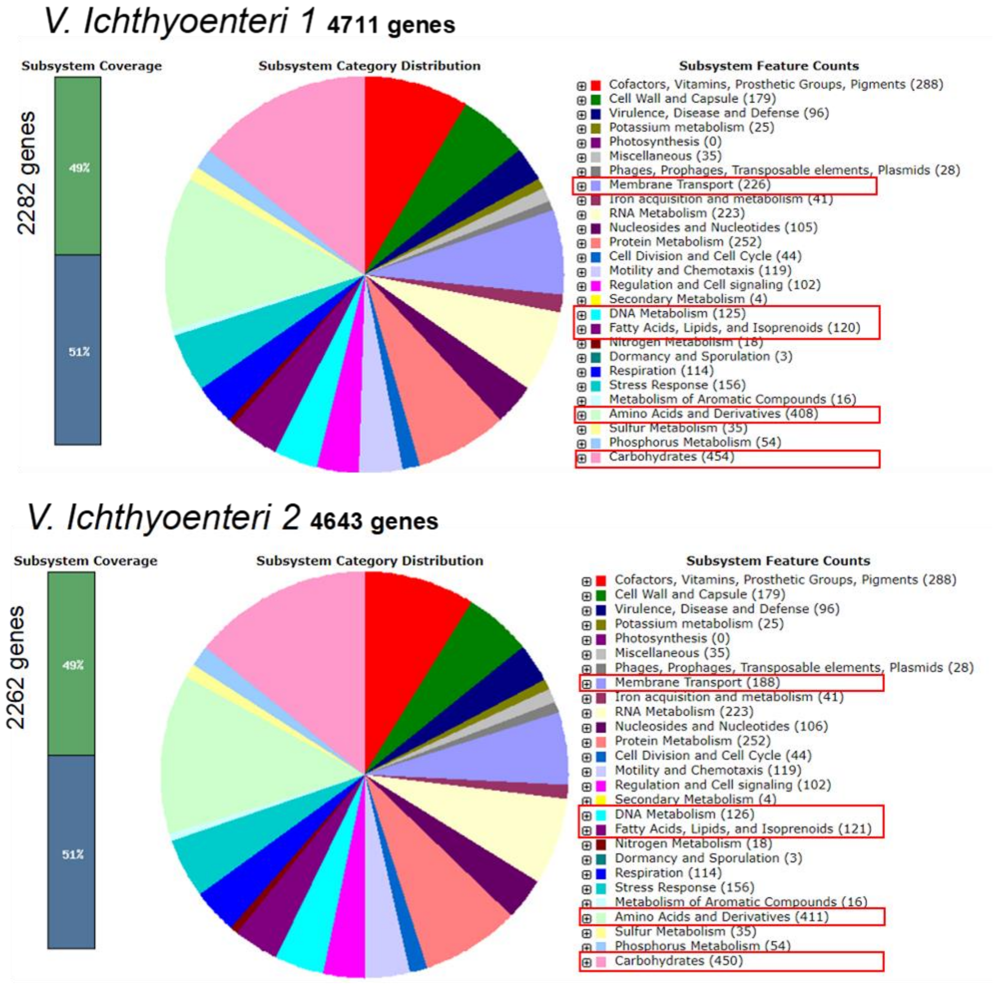


Figure 17. Pie charts representing the subsystem annotation by RAST Tool of the protein coding genes of the two *V. ichthyoenteri* strains. Annotation by RAST identified genes in *V. ichthyoenteri1* and *V. ichthyoenteri 2* and categorized them in different subsystems. The subsystems with different gene numbers are highlighted in red.

The genomic comparison with *V. ichthyoenteri ATCC 700023* (the reference strain available from the public database) revealed that most of the genomic regions are highly similar and most protein coding genes shared 99% sequence identity as revealed by the abundance of the blue-purple color in the concentric rings (Figure 18). However, differences (< 20% of the total protein coding genes) are also observed in the two isolates, indicating that the two *V. ichthyoenteri* strains are different from the reference genome and represent novel strains.

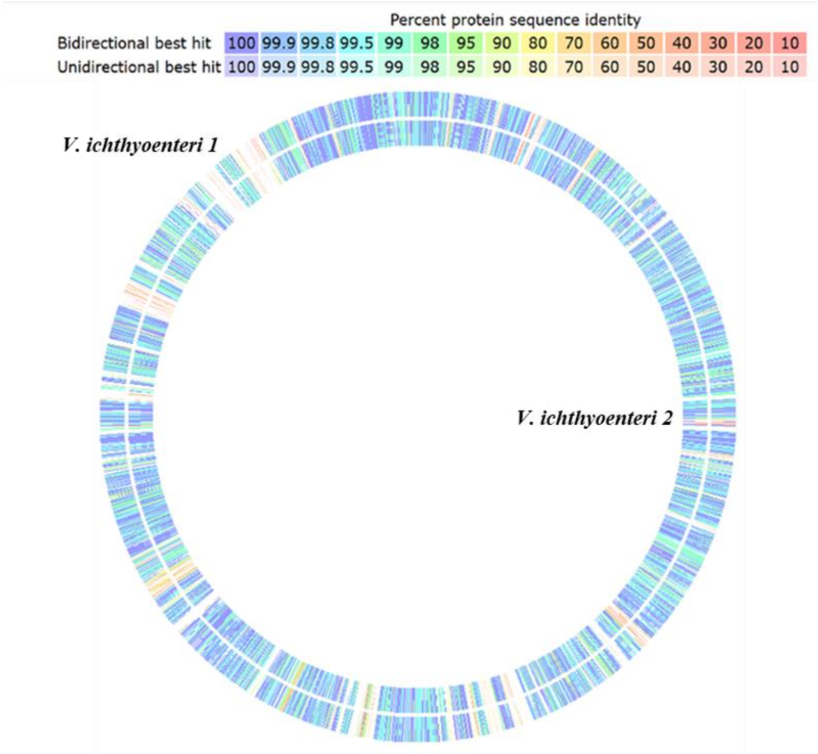
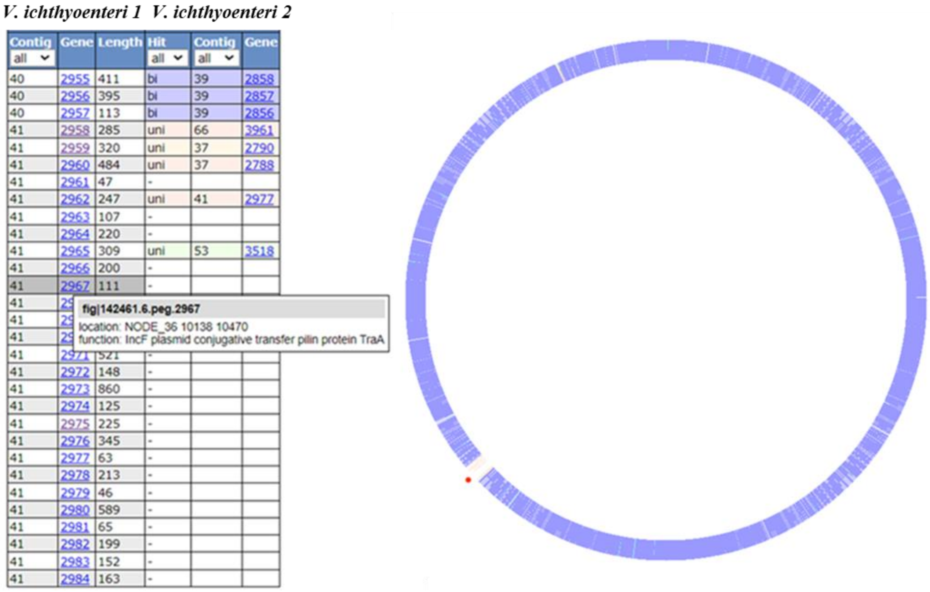


Figure 18. Graphical representation of genomic comparison among *V. ichthyoenteri* strains. *V. ichthyoenteri* ATCC 700023 was utilized as the reference strain (not depicted in the illustration). The concentric rings represent, from outer to inner: *V. ichthyoenteri* 1 and *V. ichthyoenteri* 2. The color gradients denote the amino acid similarity percentage in comparison to the reference genome, ranging from purple (100%) to light red (10%). Figure generated using the Seed Viewer sequence-based comparison tool within the RAST server.

In a separate genomic comparison, an analysis between *V. ichthyoenteri* 1 and *V. ichthyoenteri* was conducted. For this particular comparison, *V. ichthyoenteri* 1 was employed as the reference strain. The results revealed a remarkably high-percent similarity across most of their genomes except for a gap depicted as an uncoloured region (white) revealing lack of similarity (Figure 19). Digging into the uncoloured region (marked by a red circle) led to the identification of specific genes that are present in *V. ichthyoenteri* 1 but are absent in *V. ichthyoenteri 2*. This genetic segment aligns with contig node 41 of *V. ichthyoenteri 1* genome that hosts a cluster of genes characteristic of the Incompatible F (IncF) plasmid such as the genes *TraA*, *TraL*, *TraE*, *TrbC*, *TraN*, and *TraF* that play a crucial role in the construction of pili structures and other key virulence factor components in the process of plasmid conjugation(108). The full extracted comparison table along with these genes are provided in supplementary material 1. The presence of these genes in *V. ichthyoenteri* 1 and their absence in *V. ichthyoenteri* 2 highlights the possibility of plasmid-mediated conjugation in triggering horizontal gene transfer in the genome of *V. ichthyoenteri*1.



Contig all	Gene	Length	Hit all	Contig all	Gene
40	2955	411	bi	39	2858
40	2956	395	bi	39	2857
40	2957	113	bi	39	2856
41	2958	285	uni	66	3961
41	2959	320	uni	37	2790
41	2960	484	uni	37	2788
41	2961	47	-		
41	2962	247	uni	41	2977
41	2963	107	-		
41	2964	220	-		
41	2965	309	uni	53	3518
41	2966	200	-		
41	2967	111	-		
41	29				
41	29				
41	29				
41	2971	521	-		
41	2972	148	-		
41	2973	860	-		
41	2974	125	-		
41	2975	225	-		
41	2976	345	-		
41	2977	63	-		
41	2978	213	-		
41	2979	46	-		
41	2980	589	-		
41	2981	65	-		
41	2982	199	-		
41	2983	152	-		
41	2984	163	-		

fig|142461.6.peg.2967
location: NODE_36 10138 10470
function: IncF plasmid conjugative transfer pilin protein TraA

Figure 19. Genome comparison of *V. ichthyoenteri* 1 and *V. ichthyoenteri* 2 strains generated using the Seed Viewer sequence-based comparison tool within the RAST server. In this comparison, *V. ichthyoenteri* 1 was utilized as the reference strain (not depicted in the illustration) and the concentric ring represents *V. ichthyoenteri* 2. The Figure shows 100 percent similarity across most of their genomes and the main difference (marked with red circle) is noticeable as uncolored region representing the lack of *V. ichthyoenteri* 1 IncF plasmid genes in this region in *V. ichthyoenteri* 1. The Table represents the genes (as an example TraA protein) in contig 41 in *V. ichthyoenteri* 1 which are absent in *V. ichthyoenteri* 2.

3.2 The sucrose operon in the two *V. ichthyoenteri strains*

To investigate the phenotypic difference of the two isolates regarding sucrose utilization (fermenting and not fermenting) which aligns with previous study (78)on different strains of

V. ichthyoenteri, we examined in depth the genomes of both strains to search of sucrose operons that are involved in the regulation of sucrose metabolism (Figure 20).

Genes involved in sucrose utilization that are part of the sucrose operon in bacteria were found and in the two *V. ichthyoenteri* strains the operon is composed by four gene clusters like in most other bacteria (Figure 20). In the *V. ichthyoenteri* 1 genome two sucrose operons were found and in *V. ichthyoenteri 2* only a single gene cluster exists. In the *V. ichthyoenteri* 1 one sucrose gene cluster was named operon 1A, and was located in contig nodes 36 and 70, and operon 1B, was located in contig node 34. In *V. ichthyoenteri* 1 the *scrA* gene is shared between two contig nodes, specifically node 36 and node 70. Recognizing the possibility of a sequencing technical issue with the genome assembly causing this fragmentation, these separated fragments were merged as the *scrA* gene sequence was divided in the two node contigs and part predicted at the end of node 70 and the rest at the beginning of node 36. Furthermore, we observed that node 70, which contained part of the *scrA* gene, was adjacent to the *scrR* gene further supporting that they are related. Conversely, *V. ichthyoenteri* 2 harbors a single sucrose operon that is homologous in structure to the sucrose operon 1B. These homologous clusters contain the four sucrose operon genes (*scrB*, *scrK*, *scrA*, and *scrR*) with an identical organization (Figure 20). The sucrose operon 1A is also composed of the four genes but the organization is different from 1B. Interestingly, the orientation of all the sucrose operon genes in both *V. ichthyoenteri* genome contigs are located on the reverse strand, except for the repressor genes which map on the forward strand (Figure 20). The number of genes predicted by the RAST tools is higher than what was expected because in *V. ichthyoenteri*1 there were 5 genes and in *V. ichthyoenteri* 2 there were 11 genes. The additional gene copies in the operons of the two species, four in *V. ichthyoenteri* 1 and eight in *V. ichthyoenteri* 2 s were not part of the sucrose operon although considered as sucrose utilization genes.

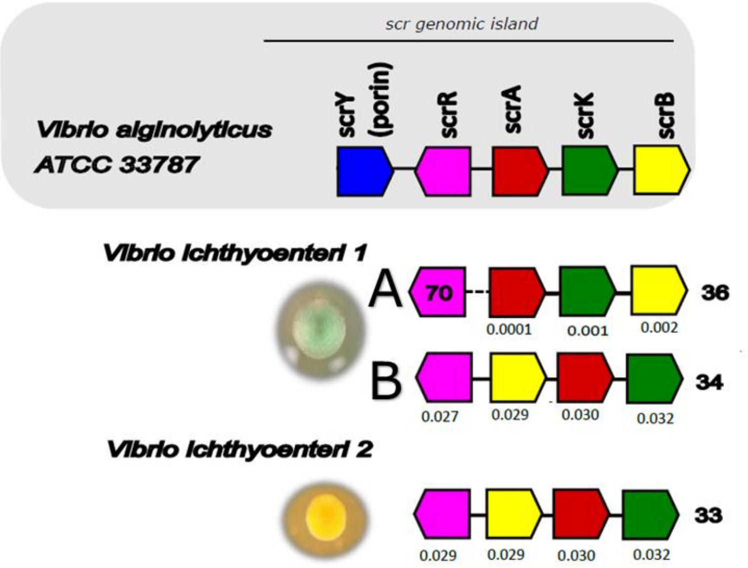


Figure 20. Organization of the sucrose operon in *V. ichthyoenteri* 1 *and V. ichthyoenteri* 2. The colored arrows represent the genes and arrow end points the direction they are in the genome. Same color indicates the same genes. There are two sucrose operons in *V. ichthyoenteri* 1: 1A and 1B. As shown in the figure the bacteria colonies in TCBS agar culture plates of *V. ichthyoenteri* 1 cannot ferment the sucrose (green color) and *V. ichthyoenteri* 2 can ferment the sucrose (yellow color) The organization of the *V. alginolyticus* was selected as reference as this was previously published (98). The porin gene (*scrY*) is not part of the classical operon but is present in many species.

3.3 Comparative analysis of sucrose operon across Vibrios

To understand the evolution of the sucrose operon in both *V. ichthyoenteri* strains they were compared to other related bacteria, available from our in-house collection and public databases (Supplementary material 3). Our analysis revealed that the in-house genomes of *V. harveyi 1, V. harveyi 2*, and *V. alfacsensis* contained all genes involved in sucrose utilization as mentioned earlier, plus the *scrR* and *scrY* genes. However, *V. parahaemolyticus* lacked all sucrose utilization genes including the *scrR* and *scrY* as expected since this bacterium does not utilize sucrose (Figure 21). The organization and orientation of the *V. ichthyoenteri* 1A operon genes are similar to the other *Vibrio* species, but totally distinct from *V. ichthyoenteri* 1B *and V. ichthyoenteri* 2, suggesting that the operons of these two bacteria have undergone a distinct evolution trajectory. Additionally, *V. renipiscarius* also possessed two sucrose operon-like gene clusters, similarly to *V. ichthyoenteri 1,* but the operon organization and sucrose fermentation are totally distinct between both as *V. renipiscarius* can utilize sucrose and *V. ichthyoenteri* 1 cannot. This difference in the sucrose utilization suggests that the sucrose operon is fully functional in *V. renipiscarius* but not in *V. ichthyoenteri* 1 despite the presence of two sucrose operons (Figure 21). Moreover, the organization of the sucrose operon in *V. renipiscarius* differs from the reference species (*V. alginolyticus*) because the first operon on node 4 lacks the *scrR* gene and the second operon on node 7 lacks the *scrK* gene. Overall, the results showed that, apart from *V. parahaemolyticus* and *V. ichthyoenteri* 1, all other *Vibrio* species were capable of metabolizing sucrose, even *V. renipiscarius* where genes of the sucrose operon are distributed in many genome contigs, and duplicate genes seem to exist.

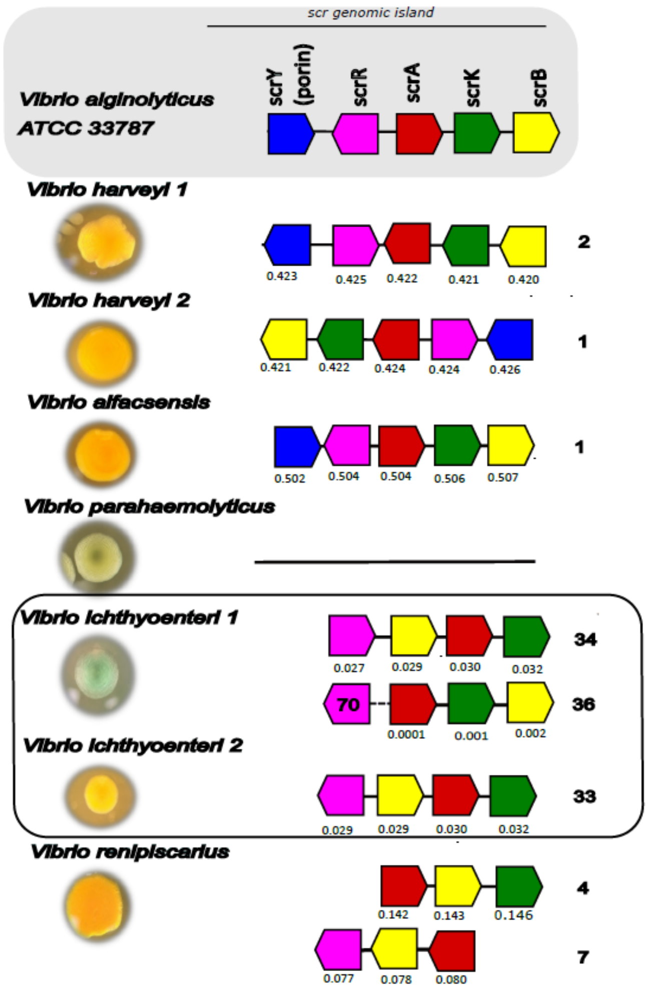


Figure 21. Comparison of the organization and orientation of sucrose operon genes (*scrA*, *scrB*, *scrK*, *scrR*) across various *Vibrio* species. Genes with the same colors indicate that they are the same genes in different species. Additionally, the presence of yellow colonies in TCBS agar plates in *V. harveyi* 1, *V. harveyi* 2, *V. alfacsensis*, *V. renipiscarius* and *V. ichthyoenteri* 2 indicates successful sucrose fermentation in these species.

3.4 Evolutionary analysis of sucrose operon genes

The individual comparisons of deduced amino acid sequences and phylogenetic analysis were carried out individually for each gene from both *V. ichthyoenteri* strains and various *Vibrio* species (Supplementary material 3). Multiple sequence alignments of the deduced proteins of *scrA*, *scrB*, *scrK*, and *scrR* genes revealed that *V. ichthyoenteri* 1A genes shares a higher sequence similarity (more than 90%) with the orthologues in *V. kanaloae* when compared with *V. ichthyoenteri* 2 *(*Table 4). In contrast all the sucrose operon genes from *V. ichthyoenteri* 1B are 100% identical to those in *V. ichthyoenteri* 2 (Table 5).

Table 4. Percentage of Sequence Similarities of the Sucrose operon genes deduced proteins between *V. ichthyoenteri 1A* with other *Vibrio* species. Comparison between the four sucrose operon genes (*scrA*, *scrB*, *scrK*, and *scrR*) is shown. The highest sequence similarity was obtained for *V. kanaloae,* and this is highlighted in bold. The percentage of sequence similarities were calculated using GeneDoc program.

	V.ichthyoenteri 1A			
	ScrA	***ScrB***	***ScrK***	***ScrR***
V. alfacsensis	86%	73%	78%	85%
V. harveyi_1	86%	73%	78%	85%
V. harveyi_2	86%	73%	78%	84
V. ichthyoenteri_2	31%	34%	63%	21%
V. ichthyoenteri_1B	31%	34%	63%	21%
V. scophthalmi	32%	33%	63%	24%
V. kanaloae	**93%**	**96%**	**98%**	**98%**

Table 5. Percentage of Sequence Similarity of deduced proteins of the Sucrose operon genes between *ichthyoenteri 2* with other *Vibrio* species. Comparison between the four sucrose operon genes (*scrA*, *scrB*, *scrK*, and *scrR*) is shown. The highest sequence similarity (100% similarity) was obtained for *V. ichthyoenteri* 1B in all four genes and after that with *V. scophthalmi*. The percentage of sequence similarities were calculated using GeneDoc program.

	V. ichthyoenteri 2			
	ScrA	***ScrB***	***ScrK***	***ScrR***
V. alfacsensis	33%	33%	64%	21%
V. harvey_1	33%	33%	65%	22%
V. harvey_2	33%	33%	65%	22%
V. ichthyoenteri_1A	31%	34%	63%	21%
V. ichthyoenteri_1B	**100%**	**100%**	**100%**	**100%**
V. scophthalmi	**96%**	**78%**	**87%**	**81%**
V. kanaloae	34%	34%	63%	21%

Phylogenetic tree of the individual genes confirmed the results from sequence identity and revealed that *scrA*, *scrB*, *scrK*, and *scrR* genes from *V. ichthyoenteri* 1A consistently cluster with good bootstrap statistical support within the homologue proteins of *V. chagasii*, *V. crassostreae,* and *V. kanaloae,* that are part of the Splendidus clade instead of clustering with its evolutionary related bacteria in the Scophthalmi clade (Supplementary material 3). On the other hand, all the genes that are part of the sucrose operon in *V. ichthyoenteri* 1B and *V. ichthyoenteri* 2 consistently cluster together and within the Scophthalmi clade with *V. scophthalmi*. The different clustering of the sucrose operon genes of *V. ichthyoenteri* 1A and *V. ichthyoenteri* 1B suggests that they have evolved differently (Supplementary material 3).

To better understand the evolution of the sucrose operons in relation to the proposed *Vibrio* species evolution we conducted a parallel phylogenetic analysis with several *Vibrio* species where sucrose operons have been characterized (Figure 22). The phylogenetic tree that resulted from the concatenated sequences of the sucrose operon genes revealed a different pattern of evolution for *V. ichthyoenteri* 1A as expected based on single gene analysis and the clustering within the Splendidus clade. The other *V. ichthyoenteri* operon grouped with the phylogenetically related *V. scophthalmi* in phylogenetic trees of the sucrose genes and this agrees with the proposed species tree. The close association of *V. ichthyoenteri* 1A and the Vibrios that are members of Splendidus clade is enigmatic. To better understand evolution of the sucrose operon the neighboring genes were characterized.

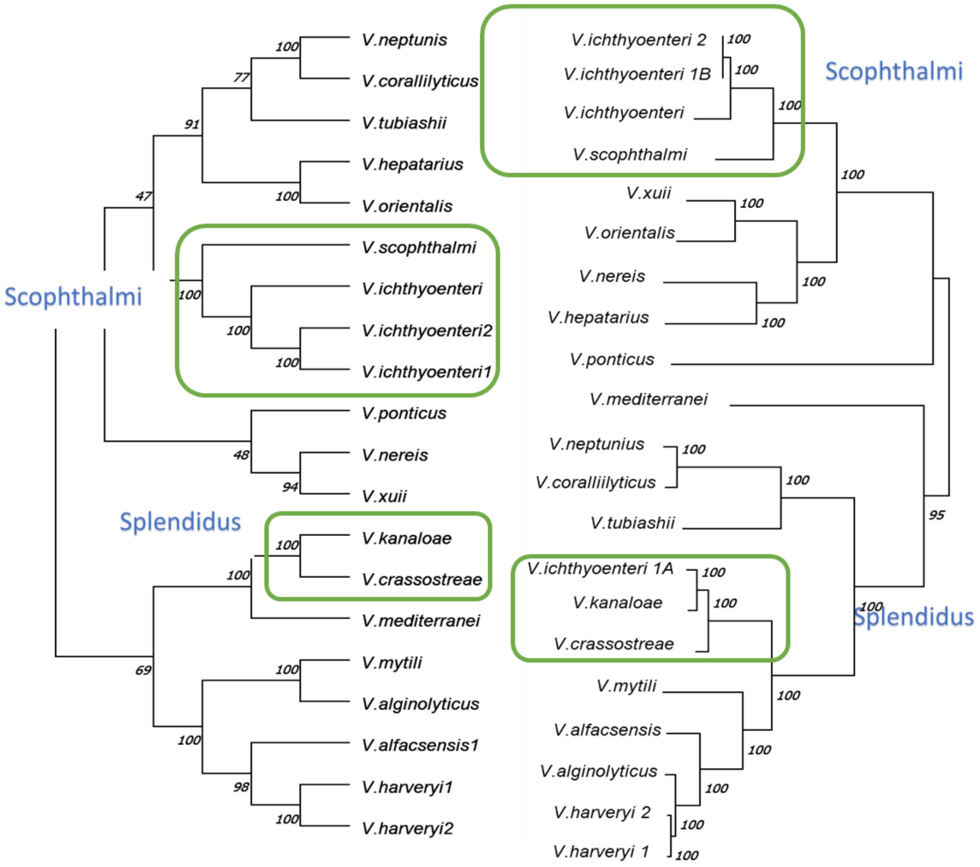


Figure 22. Species and sucrose operon phylogenetic analysis. On the left side, is represented the species tree and on the right side displays the phylogenetic tree of the sucrose operon genes. Both were carried out by using the Maximum Likelihood method with Mega 11. The members of the Splendidus and Scophthalmi clades are indicated by the green boxes.

3.5 Gene synteny analysis of the sucrose operons in *V. ichthyoenteri*

To understand how genes might have potentially evolved, the neighboring genes that flank the sucrose operon in both *V. ichthyoenteri* strains were retrieved and homologue genome regions were identified. Analysis revealed that the sucrose operon genes in *V. ichthyoenteri* 1A evolved differently from *V. ichthyoenteri* 1B (Figure 23).

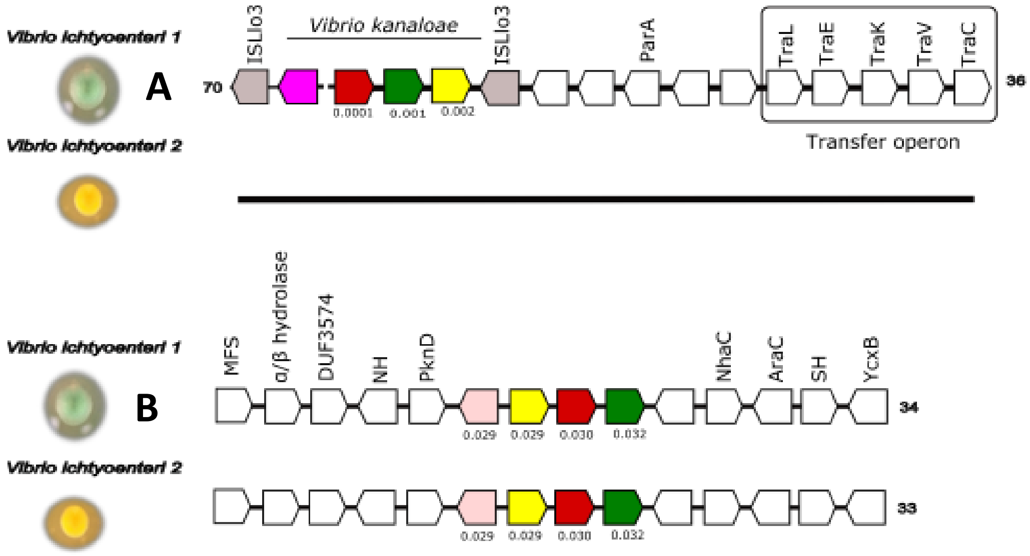


Figure 23. Characterization of the gene environment surrounding the sucrose operon loci of two *V. ichthyoenteri* strains. *V. ichthyoenteri* 1B, and *V. ichthyoenteri* 2, along with a comparison to the sucrose operon locus of *V. ichthyoenteri* 1A. The genes are visually represented by arrow boxes, and the orientation of the arrow ends signifies the gene orientation within the genome. The genes related to the sucrose operon are highlighted in specific colors, and matching colors denote homologous genes. The remaining genes are colored differently. Gene symbols that are known are labeled above the respective arrows.

In both *V. ichthyoenteri* 1B and *V. ichthyoenteri* 2, the gene environment surrounding their respective sucrose operon loci is identical, with 23 genes upstream and 19 genes downstream of the sucrose operon locus. This remarkable similarity in both *V. ichthyoenteri* 1B and *V. ichthyoenteri* 2, implies a potential common evolutionary history for this operon in these two strains. However, the sucrose operon locus of *V. ichthyoenteri* 1A presents a totally different gene environment. No orthologues of the genes found in *V. ichthyoenteri* 1B and *V. ichthyoenteri* 2 were identified in the sucrose operon locus of *V. ichthyoenteri* 1A. Instead, the analysis of the neighboring genes revealed the presence of gene clusters typical of Plasmid F, such as the transfer cluster. The F-plasmid, a circular conjugative plasmid found in *Escherichia coli*, was first recognized as a vector for horizontal gene transfer and gene recombination in the late 1940s. This plasmid enables the transfer of genes from a bacterium possessing the F-factor (F+) to another bacterium lacking it (F-) through conjugation process (108–110). This observation suggests that the sucrose operon in *V. ichthyoenteri* 1A might have originated from a different source compared to that of *V. ichthyoenteri* 1B and *V. ichthyoenteri* 2. The presence of Plasmid F-related gene clusters in the vicinity of the sucrose operon locus of *V. ichthyoenteri* 1A raises the possibility of horizontal gene transfer events involving this operon that may have been transferred from a bacteria that is a member of the Splendidus clade to which it shares the highest sequence similarities. The similarity between the *V. ichthyoenteri* 1A with the genes of other Vibrios from the Splendidus clade suggests that this gene cluster may have been transferred for a member of the Splendidus clade to *V. ichthyoenteri* 1 via bacterial conjugation. The conjugation process starts with connecting the pilus on the surface of the F+ cell to the recipient cell, leading to the formation of a mating junction(111). This interaction initiates the cleaving, unwinding, and subsequent transfer of DNA to the recipient cell, thereby facilitating the horizontal transfer of genetic material(112). It is plausible to suggest that the inactivity of the sucrose operon in *V. ichthyoenteri* 1 could be attributed to the presence of the F-plasmid where an extra copy of the operon is encoded. However, how the presence of an extra copy gene for sucrose utilization that comes from another species that is in principle functional interferes with the functionality of the already existent operon is not understood. Further investigation is necessary to confirm this hypothesis and elucidate the specific mechanisms underlying this phenomenon.

3.6 Functional characterization

To characterize the potential functional differences related to sucrose fermentation in *V. ichthyoenteri*, the growth profile of the two different strains, the activity of proteolytic and hydrolytic enzymes, and the analysis of the expression of metabolizing sucrose genes were characterized and compared.

3.6.1 Effect of sucrose on growth profile

Both strains exhibit a very similar growth in TSB/1% NaCl (Figure 24). The isolated *V. ichthyoenteri* 1 showed a growth rate of 0.46 ± 0.01 h^{-1} and doubling time (dt) of 1.47 ± 0.05 h and *V. ichthyoenteri* 2 had a growth rate of 0.47 ± 0.026 h^{-1} and dt of 1.45 ± 0.07 h. In the peptone medium also both strains exhibited a similar growth behavior regardless the presence or absence of sucrose. Although *V. ichthyoenteri* 2 possesses the capability to metabolize sucrose, the presence of this carbohydrate in the peptone medium does not appear to contribute to bacterial growth. This observation is evident as the growth curve of *V.*

ichthyoenteri 2, which in the presence of sucrose fails to reach the exponential phase, and resembles the behavior of *V. ichthyoenteri* 1, which does not metabolize sucrose (Figure 24). This suggests that when sucrose is available, it does not modify bacteria growth and that perhaps utilization of sucrose may be involved in *V. ichthyoenteri* the production of secondary metabolites but this requires further studies.

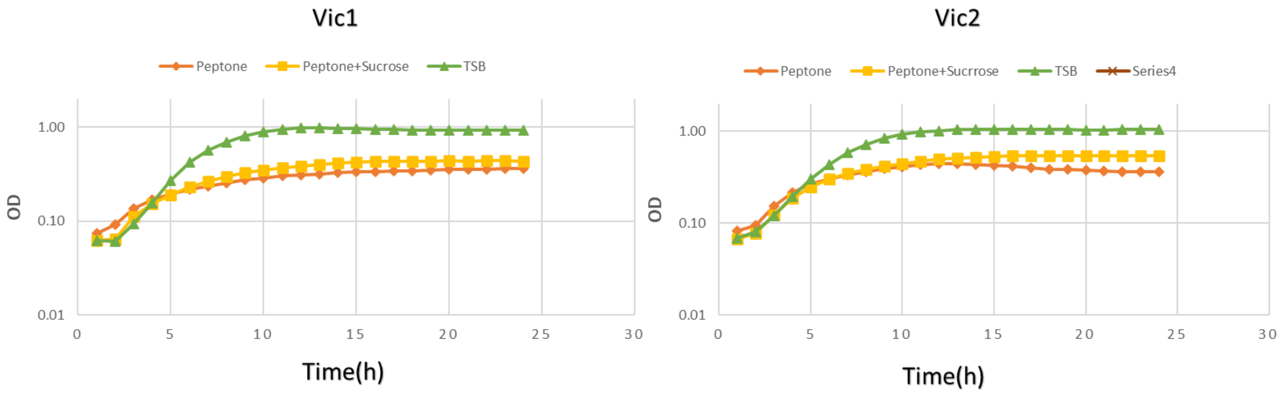


Figure 24. Growth curves of *V. ichthyoenteri* strains in various culture media. The figure illustrates the growth patterns of both *V. ichthyoenteri* strains in three distinct culture media: peptone medium, peptone medium supplemented with sucrose, and Tryptic Soy Broth (TSB). Remarkably, both strains exhibit robust growth when cultivated in TSB. Interestingly, the growth behavior of both *V. ichthyoenteri* 1 and *V. ichthyoenteri* 2 remains similar in peptone medium, irrespective of the presence or absence of sucrose supplementation. Notably, although *V. ichthyoenteri* 2 possesses the capability to metabolize sucrose, this carbohydrate does not appear to significantly contribute to bacterial growth in the context of peptone medium. Vic1 indicates *V. ichthyoenteri* 1 and Vic2 indicates *V. ichthyoenteri* 2

3.6.2 Effect of sucrose on extracellular enzyme activity

To assess the potential association of the carbohydrate source and the potential expression of virulence related genes, the activity of two extracellular enzymes, hydrolases (esterase) and proteases, was characterized under the presence and absence of sucrose (Figure 25).

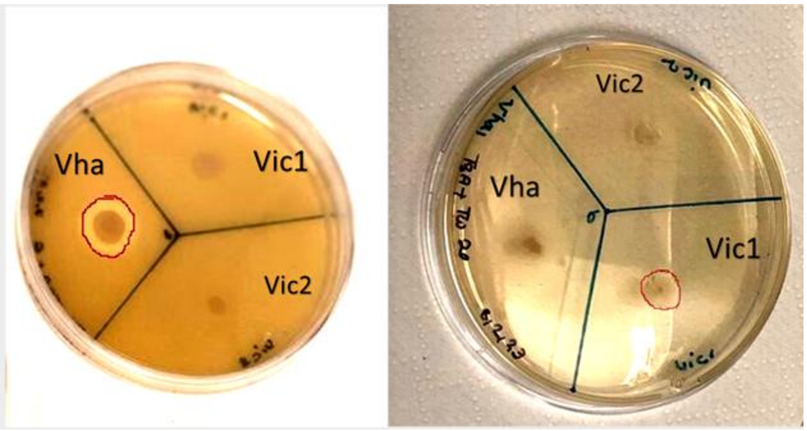


Figure 25. Digital photographs of the agar plates showing the protease activity (left) esterase activity (right) of *V. ichthyoenteri strains* and *V. harveyi*. Incubations were performed at 22 ºC for 48h. The presence of protease and esterase activity is indicated by the appearance of a halo that is demarcated by the red circle. Vic1 indicates *V. ichthyoenteri* 1, Vic2 indicates *V. ichthyoenteri* 2and Vha indicates *V. harveyi*.

The results of the esterase activity assay showed significant differences in the enzyme activity of the two *V. ichthyoenteri* strains and the positive control *V. harveyi* with and without the presence of sucrose. When sucrose in not present esterase activity was observed in *V. ichthyoenteri* 1 (EI= 1.6), *V. ichthyoenteri* 2 (EI= 2.8), and *V. harveyi* (EI= 5.8) (Figure 26). However, when 1% sucrose was added to the culture medium, no esterase activity was detected in any of the three bacteria strains. The absence of esterase activity suggests that sucrose may exert an inhibitory effect on the expression or activity of esterase enzymes. The result of esterase activity observed in *Vibrio* species in the presence of sucrose appears to be comparable to the effect of supplying sucrose as a carbohydrate to *V. cholerae* where it dampens down the production of the cholera toxin (102). Both phenomena demonstrated a reduction of the expected activity with the presence of sucrose, indicating a potential connection between the use of sucrose as a carbohydrate source and the decrease expression of potential virulence factors by pathogenic bacteria.

In terms of proteolytic behavior, no detectable activity was observed in *V. ichthyoenteri 1 and V. ichthyoenteri 2*, regardless of the presence or absence of sucrose. Interestingly, the addition of 1% sucrose to the culture medium led to a decrease in protease activity in *V. harveyi,* however this decrease was not statistically significant. The enzyme index decreased from EI= 1.7 to EI= 1.3, suggesting that sucrose negatively affected the production of protease enzymes in this strain. Sucrose may interfere with protease synthesis, secretion, or enzyme-substrate interactions, resulting in the observed reduction in protease activity. These findings suggest a potential connection between enzyme activity and carbohydrate source in bacterial systems (113–115) that require to be further elucidated.

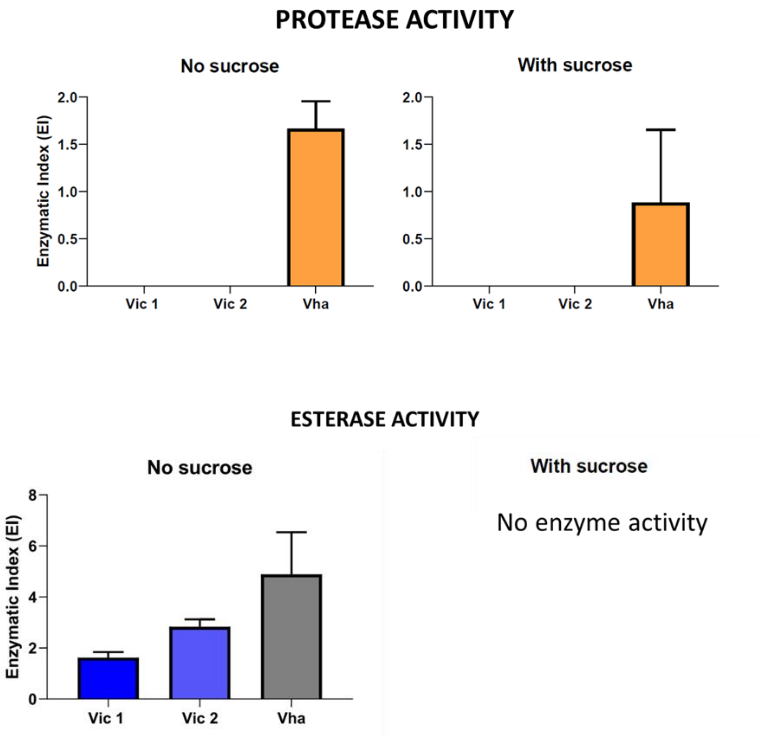


Figure 26. Comparative Analysis of Hydrolase (Esterase) and Protease Secretion in the two *V. ichthyoenteri* strains and *V. harveyi*. The figure illustrates enzyme indexes representing the secretion of hydrolases (esterase) and proteases in the presence and absence of sucrose. *V. harveyi* was chosen as a positive control. Vic1 indicates *V. ichthyoenteri* 1, Vic2 indicates *V. ichthyoenteri* 2and Vha indicates *V. harveyi*.

3.6.3 Effect of sucrose on gene expression

To characterize the effect of presence of sucrose in gene expression of the sucrose operon genes the initial step involved the extraction of RNAs. Based on the results observed (Figure 27), the presence of distinct and well-defined 23S and 16S rRNA bands in the RNA extraction indicates that the overall quality of the RNA samples is likely to be good. The appearance of these bands without smearing or degradation suggests that the extracted RNA is of high integrity. Total RNA was used for cDNA synthesis and due to the lack of triplicates for the samples, quantitative PCR (qPCR) could not be performed to determine the gene expression levels. Instead, conventional qualitative PCR was carried out to assess whether the expression of the target genes was stimulated or not by the presence of sucrose in the culture medium. To understand functional regulation of the sucrose operon in the presence of sucrose we have analyzed the expression of the *scrB* gene which is common between the sucrose metabolizing (*V. ichthyoenteri* 2) and non-metabolizing (*V. ichthyoenteri*1) strains.

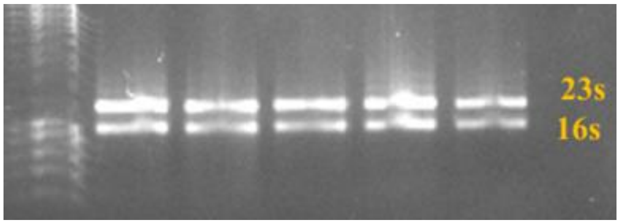


Figure 27. Agarose Gel Electrophoresis of total RNA Samples from *V. ichthyoenteri1 and V. ichthyoenteri2* Strains. Agarose gel electrophoresis of tRNA from 5 samples results from the left: 1) *V. ichthyoenteri* 1 in peptone at 4hours 2) *V. ichthyoenteri* 1 in peptone medium supplemented with sucrose at 4hours. 3) *V. ichthyoenteri* 2 in peptone at 4hours 2) *V. ichthyoenteri* 2 in peptone medium supplemented with sucrose at 4hours 5) *V. ichthyoenteri* 1 in peptone at 8hours.*M*- Molecular *ladder* NZYTech Ladder III (10000-200 bp)

The PCR results obtained demonstrated a strong and consistent pattern, with all bands being clearly observable suggesting that *scrB* gene was expressed in all samples irrespective of the presence and absence of sucrose and in both *V. ichthyoenteri* strains including the one (Vic1) where the sucrose operon is apparent non-functional (Figure 28). This is a puzzling observation as it was expected that no gene expression would occur because this bacterium cannot metabolize sucrose as was revealed by the TCBS plates, but the operon genes seem to be expressed irrespectively. To assess potential differences in gene relative expression the intensity of the PCR products for *scrB* genes were normalized with the 16S expression (Figure 29).

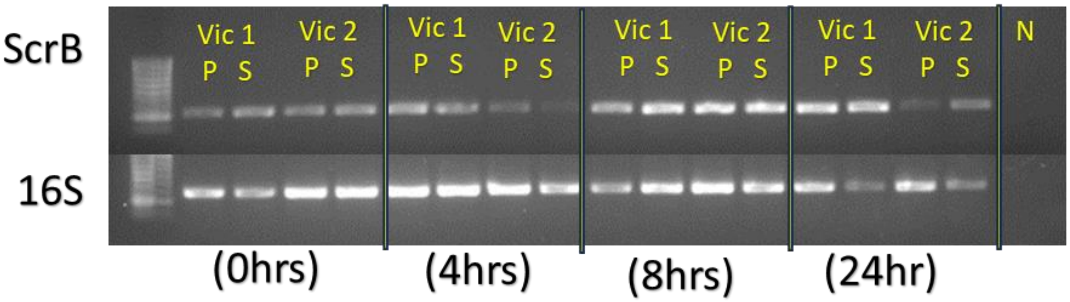


Figure 28. Gel electrophoresis of the PCR results from amplification of scrB and 16S using cDNA obtained from *V. ichthyoenteri* strains cultured in the presence and absence of sucrose at various time points. The upper panel displays the PCR products of the *16S* gene, while the lower panel shows the PCR products of the *scrb* gene. Vic1 indicates *V. ichthyoenteri*1 and Vic2 indicates *V. ichthyoenteri*2 and "P" and "S" signify the culture media employed, with "P" denoting peptone medium and "S" indicating peptone medium supplemented with sucrose and N is for negative control reaction.

Relative expression analysis showed that almost in all samples with sucrose supplementation the gene *scrB* was slightly more expressed when compared to the samples without sucrose supplementation in both strains (Figure 29). This observation suggests that as expected the presence of sucrose induced expression of sucrose metabolizing genes, in this case the *scrB* that is responsible for sucrose hydrolysis and the levels of gene expression in the absence of sucrose revealed that there might be a basal constitutive expression. Moreover, there seems to be a gradual increase in the expression level of the s*crB* gene product as the culture time progresses in all samples as revealed in the 8 hours and 24 hours samples (Figure 29).

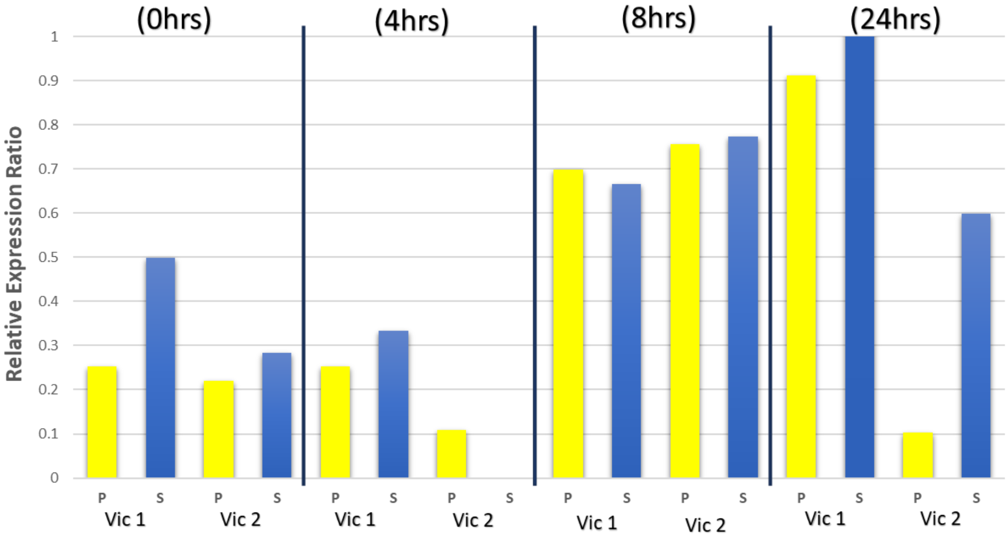


Figure 29. Graph of Relative Expression Comparison. Obtained from dividing the ScrB quantity (detected by program) by 16 quantities (detected by program)

This increased gene expression even in the culture media that does not contain any kind of carbohydrate suggest that expression of *scrB* genes may occur under conditions beyond the presence of any carbohydrate source and may probably be involved in other metabolic processes or regulatory pathways that are not directly related to sucrose metabolism.

Considering that sucrose utilization is not solely dependent on *scrB* genes therefore, it is likely that *V. ichthyoenteri1* may lack other necessary components or factors in the sucrose metabolic pathway. Another fact may be that the protein ScrB produced lacks its biological function and thus is not able to hydrolyze the sucrose. During this study there were samples of the culture medium that were collected to measure the glucose levels however due to time constrains this was not possible and it would be interesting in the future to correlate genes expression with the changes in the production of glucose (that resulted from sucrose degradation) levels across time. An additional explanation could be related to the presence of plasmid-derived sucrose operon. It's plausible that the existence of such operons, possibly originating from Plasmid F, might play a role in the variation of sucrose utilization abilities between strains. The existence and potential influence and crosstalk of a second sucrose operon similar to the functional operons present in most bacteria and in other *Vibrio* species in gene regulation and protein function of the sucrose operon in *V. ichthyoenteri* 1 is not known and requires to be further studied. Further analysis and experiments, including qPCR, would be necessary to validate the initial PCR results and gain a deeper understanding of the regulatory mechanisms and the role of sucrose in the genetic expression of the target gene.

4 Conclusion

In this study two novel *V. ichthyoenteri* strains were characterized at the genomic and phenotypic levels. Genome annotation revealed that the main difference in both strains is related to the number of genes involved in functional/metabolic processes and that in the genome of *V. ichthyoenteri*1 several genes that are characteristic of Plasmid F, the main DNA vector involved in transfer of DNA between bacteria. The two strains have different numbers of sucrose operon clusters and *V. ichthyoenteri* 1 has two distinct sucrose operons (A and B), with operon B being identical to that in *V. ichthyoenteri* 2 and this is likely to explain the observed phenotypic differences in the sucrose utilization assays. Bioinformatic analysis revealed that *V. ichthyoenteri* 1A shared greater genome structure and sequence identity with other *Vibrio* species (splendidus clade) while *V. ichthyoenteri* 1B and *V. ichthyoenteri* 2 were identical and similar to *Vibrio* of the Scophthalmi clade. Gene linkage analysis revealed the presence of Plasmid F-related gene clusters in the vicinity of the sucrose operon locus of *V. ichthyoenteri* 1A posing the possibility of horizontal gene transfer (HGT) events involving this operon from a bacterium that is member of the Splendidus clade to which it shares the highest sequence similarities. Growth kinetics failed to reveal differences between the two *V. ichthyoenteri* strains in the presence of sucrose, indicating that it does not influence the growth of both bacteria, but sucrose is likely to be metabolized by *V. ichthyoenteri 2* as evidenced by color changes in TCBS and phenol red assays. Biochemical assessments revealed the presence of sucrose seems to decrease or inhibit the activity of the secreted Esterase and Proteases enzymes which are related with *Vibrio* species virulence. PCR results revealed the expression of the sucrose utilization gene *scrB*, and its gradual increase even in carbohydrate-free conditions (media containing only peptone) suggesting potential adaptive responses in these *Vibrio* species. However, the increased expression of *scrB* in *V. ichthyoenteri*1 is intriguing. Considering, that elevated gene expression of the *scrB* gene alone does not guarantee efficient sucrose utilization, even when the gene produces functional protein, it is possible that the strain lacks essential components or factors necessary for this metabolic pathway. This deficiency could potentially be linked to the presence of gene clusters associated with Plasmid F.

5 Future Perspectives

Whole-Genome Sequencing with Specific Detection of Separate Chromosomes and Plasmids: In future research, conducting whole-genome sequencing with the ability to differentiate between chromosomes and plasmids, particularly the detected Incompatible Plasmid F, gives great information. This approach can provide deeper insights into the *V. ichthyoenteri* genome of both strains, allowing us to determine whether the sucrose utilization genes of operon A in *V. ichthyoenteri 1*, are placed within Plasmid F or integrated into the chromosomal DNA. This knowledge is crucial to understand the genetic organization and potential mobility of these genes.

Virulence Testing: Future investigations should involve virulence testing, to detect any differences in pathogenicity between the two *V. ichthyoenteri* strains. Despite the identical numbers and types of virulence genes identified by the RAST tool, the presence of virulence genes related to incompatible Plasmid F suggests that their expression and impact on pathogenicity may differ. This will help elucidate the precise role of Plasmid F in virulence.

Sucrose Hydrolysis Assessment: In the future, it is wise to assess sucrose hydrolysis in cultures grown for at least 24 hours with sucrose as the sole carbohydrate source for both *V. ichthyoenteri* strains. This investigation will help determine the extent to which sucrose is hydrolyzed into glucose and fructose by the *scrB* gene. Additionally, it will provide insights into the functional integrity of the protein encoded by *scrB* and reveal if this protein of the sucrose operon in *V. ichthyoenteri 1* is functional or not.

Quantitative PCR for Regulatory Insights: For a deeper understanding of the regulatory mechanisms governing sucrose utilization, future research should employ quantitative PCR (qPCR) experiments. These experiments will enable a more comprehensive analysis of how sucrose influences the genetic expression of target genes.

Studies on Horizontal Gene Transfer through Conjugation: To gain further insights into the mechanisms of horizontal gene transfer (HGT) within *V. ichthyoenteri*, future experiments under favorable conditions, such as facilitating conjugation between F+ and F- *V. ichthyoenteri* strains, should be considered. This will provide valuable information on whether incompatible Plasmid F can indeed mediate HGT between these strains and offer a more comprehensive understanding of genetic exchange mechanisms within this bacterial species.

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7 Supplementary Tables

7.1 Supplementary Material 1

Table 6. Genes extracted from Contig 41 of *V. ichthyoenteri 1* and their percentage Sequence Similarities with *V. ichthyoenteri 2*. Genes in *V. ichthyoenteri* 1 associated with the Incompatible F (IncF) plasmid that participate in the plasmid conjugation process which are not present in *V. ichthyoenteri* 2

Contig	**Gene**	**Length**	**Gene id**	**Percent identity**
41	2967	111	IncF plasmid conjugative transfer pilin protein TraA	0
41	2968	103	IncF plasmid conjugative transfer pilus assembly protein TraL	0
41	2969	190	IncF plasmid conjugative transfer pilus assembly protein TraE	0
41	2970	246	IncF plasmid conjugative transfer pilus assembly protein TraK	0
41	2971	521	IncF plasmid conjugative transfer pilus assembly protein TraB	0
41	2972	148	IncF plasmid conjugative transfer pilus assembly protein TraV	0
41	2973	860	IncF plasmid conjugative transfer pilus assembly protein TraC	0
41	2974	125	hypothetical protein	0
41	2975	225	IncF plasmid conjugative transfer pilus assembly protein TraW	0
41	2976	345	IncF plasmid conjugative transfer pilus assembly protein TraU	0
41	2977	63	hypothetical protein	0
41	2978	213	IncF plasmid conjugative transfer protein TrbC	0
41	2979	46	hypothetical protein	0
41	2980	589	IncF plasmid conjugative transfer protein TraN	0
41	2981	65	hypothetical protein	0
41	2982	199	IncF plasmid conjugative transfer pilus assembly protein TraF	0
41	2983	152	hypothetical protein	0
41	2984	163	IncF plasmid conjugative transfer protein TrbB	0

41	2985	463	IncF plasmid conjugative transfer pilus assembly protein TraH	0
41	2986	927	IncF plasmid conjugative transfer protein TraG	0
41	2987	165	hypothetical protein	29.19
41	2988	691	IncF plasmid conjugative transfer protein TraD	0
41	2989	252	IncF plasmid conjugative transfer surface exclusion protein TraT	0
41	2990	62	hypothetical protein	0
41	2991	81	hypothetical protein	0
41	2992	68	hypothetical protein	53.02
41	2993	1983	IncF plasmid conjugative transfer DNA-nicking and unwinding protein TraI	50
41	2994	95	hypothetical protein	0
41	2995	114	Plasmid related protein	0

7.2 Supplementary Figures

Supplementary Material 2 (Phylogenetic trees based on protein sequences of the (*scrA, scrB, scrA, scrA)* genes)

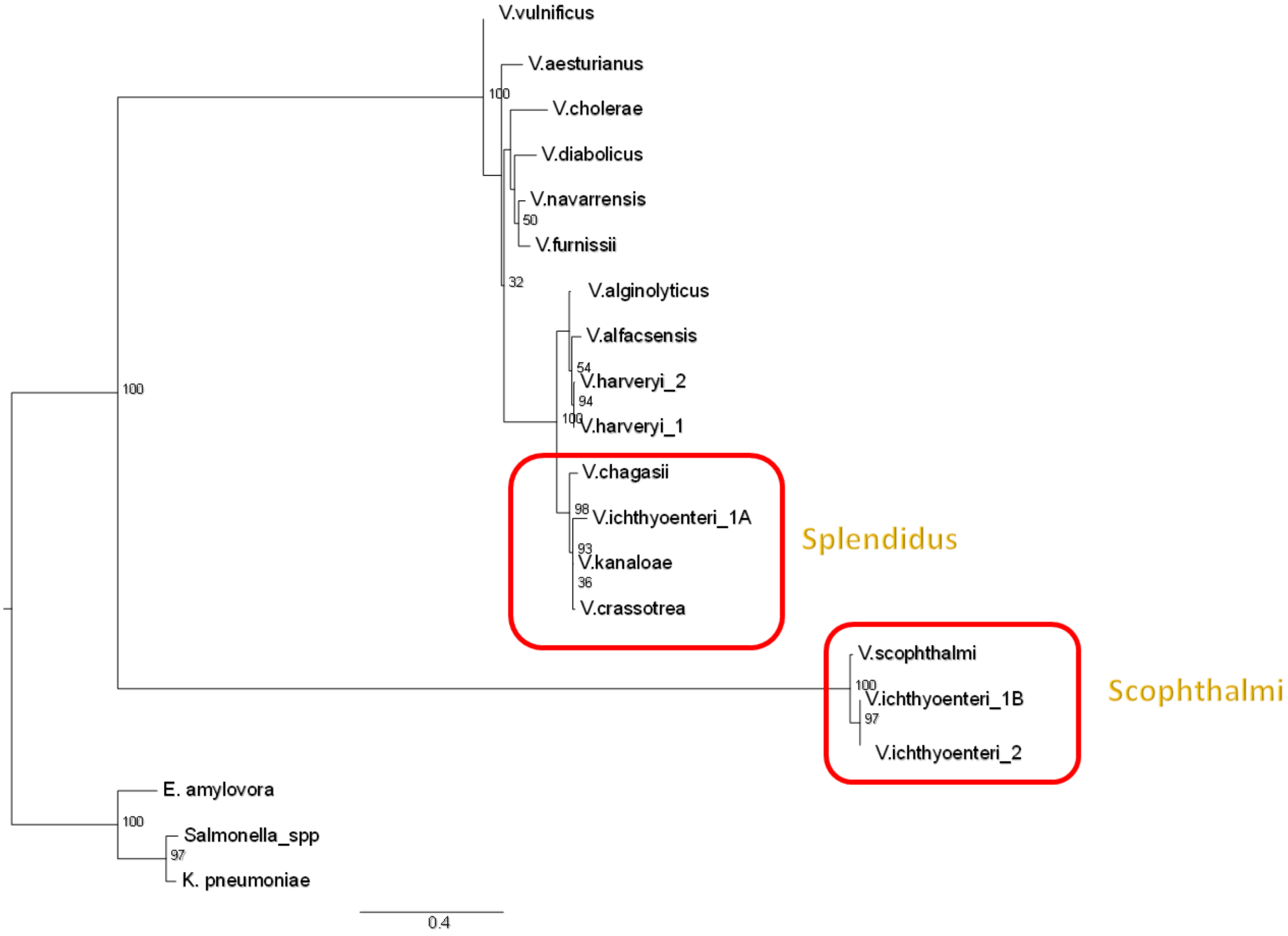

Figure 30. Phylogenetic tree based on protein sequences of the gene *scrA*. The genes from *V. ichthyoenteri* 1B and *V. ichthyoenteri* 2 consistently cluster with the Scopthalmi clade. Furthermore, the genes from *V. ichthyoenteri* 1A are situated within the Splendidus clade.

Figure 31. Phylogenetic tree based on protein sequences of the gene *scrB.* The genes from *V. ichthyoenteri* 1B and *V. ichthyoenteri* 2 consistently cluster with the Scopthalmi clade. Furthermore, the genes from *V. ichthyoenteri* 1A are situated within the Splendidus clade.

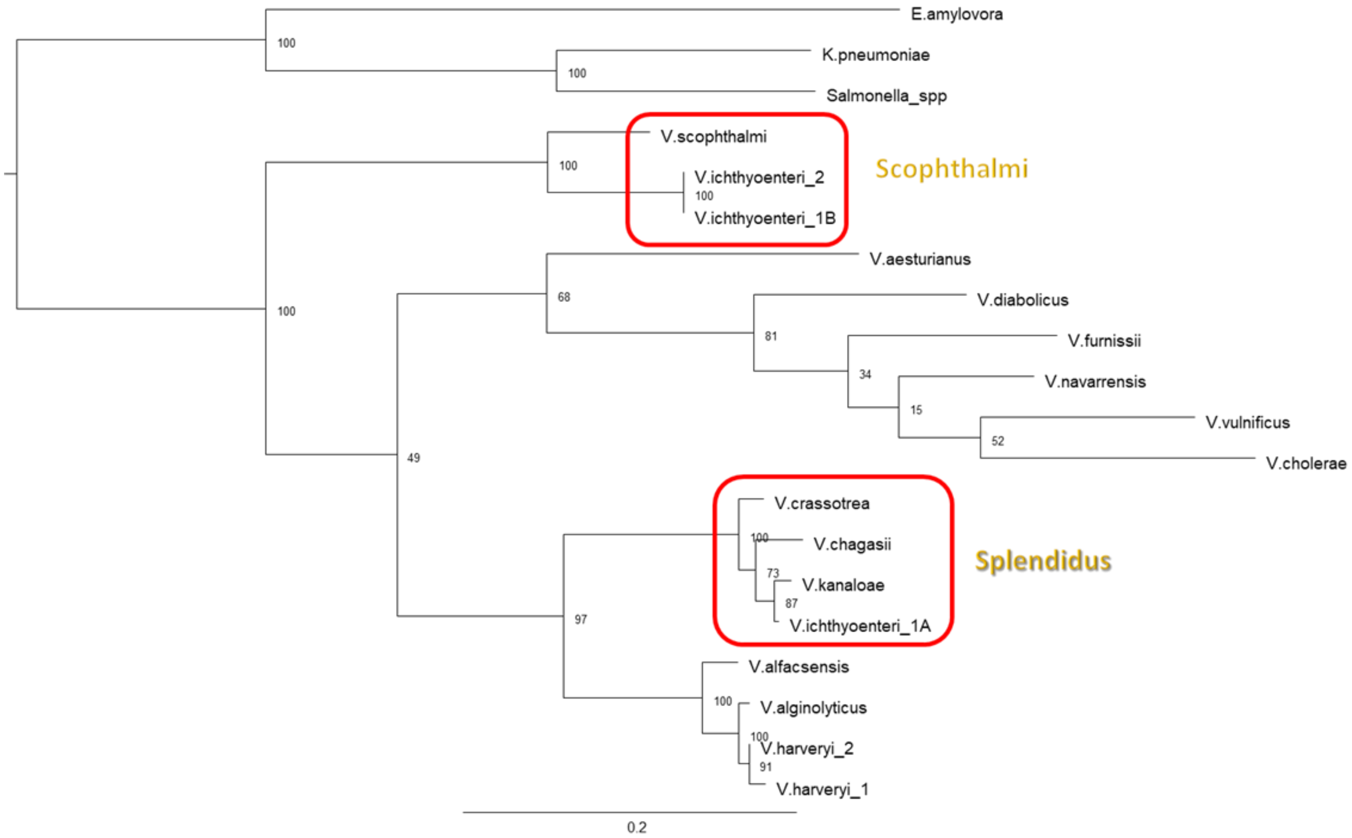

Figure 32. Phylogenetic tree based on protein sequences of the gene *scrK*. The genes from *V. ichthyoenteri* 1B and *V. ichthyoenteri* 2 consistently cluster with the Scopthalmi clade. Furthermore, the genes from *V. ichthyoenteri* 1A are situated within the Splendidus clade.

Figure 33. Phylogenetic tree based on protein sequences of the gene *scrR*. The genes from *V. ichthyoenteri* 1B and *V. ichthyoenteri* 2 consistently cluster with the Scopthalmi clade. Furthermore, the genes from *V. ichthyoenteri* 1A are situated within the Splendidus clade.