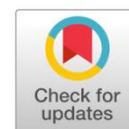




Original Research



Molecular identification of pathogenic bacteria causing foodborne disease in Caulerpa racemosa



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Abstract: *Caulerpa racemosa* is a green algae consumed by people in northern coastal areas. *C. racemosa* has a habitat attached to the shallow seabed. *C. racemosa* usually consumed fresh without any cooking process so that the contamination of microorganisms can be eaten. Molecular identification using 16S rRNA is needed to determine the type of bacterial contaminants in *C. racemosa*. The isolates of *C. racemosa* were cultured in HIA, BAP, and BHI media. Bacteria from BHI media were isolated by DNA, PCR for 16S rRNA gene, and sequencing. Bacteria isolate *C. racemosa* was found to have the α -hemolytic ability in BAP media. The sequencing analysis showed that the three bacterial colonies of *C. racemosa* isolate had high similarity with *V. parahemolyticus*, *Caldalkalibacillus mannanilyticus*, and *Exiguobacterium profundum*.

Keywords: Bacteria identification; Caulerpa racemose; Foodborne disease.

INTRODUCTION

Bacterial growth, especially in waters, is often associated with or contaminating marine organisms, one of which is *C. racemosa*.¹ The presence of bacteria can threaten the hygiene of marine biota and even human health who consume polluted marine biota.² Bacterial identification is needed to find out what types of bacteria live in symbiosis with *C. racemosa*. Identification of pathogenic bacteria is crucial to provide information regarding the safety of *C. racemosa* when consumed raw. BAP media is an enriched and differential selective medium that can differentiate pathogenic bacteria based on their ability to hemolysis erythrocytes.³ Microbiological identification of bacteria has the disadvantage of taking a long time from bacterial culture to biochemical tests.⁴

In addition to the culture method, identification of bacteria can be carried out using a molecular-based method with a high level of sensitivity by analysis using the 16S rRNA gene (16S ribosomal Ribonucleic acid / Ribonucleic acid encoding the 16S ribosome, S represents Svedberg, which is the unit of measurement for ribosomes). The gene encoding rRNA is the most conserved gene, so the 16S rRNA gene is used as a universal primer in the use of Polymerase Chain Reaction and determination of nucleotide sequences through sequencing (PCR).⁵

There is still limited research related to identifying pathogenic bacteria that cause foodborne disease in *C. racemosa*, it is necessary to research to provide new insights to the public regarding hygiene in the consumption of fresh

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DOI: [10.29238/teknolabjournal.v10i1.276](https://doi.org/10.29238/teknolabjournal.v10i1.276)

Received 13 June 2021; Received in revised form 28 June 2021; Accepted 23 July 2021

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C. racemosa. Molecular identification of bacteria causing foodborne disease in *C. racemosa* have high sensitivity and specificity regarding the types of bacteria. The 16S rRNA gene was chosen because all bacteria have this gene, so it is possible to find new species that are pathogenic and specifically found in *C. racemosa*

MATERIAL AND METHOD

A sampling of *C. racemosa* was carried out in the waters of Jepara, Central Java, Indonesia. Isolation to obtain pure bacteria from *C. racemosa* culture was carried out at the Microbiology Laboratory. In contrast, DNA isolation from *C. racemosa* culture bacteria and molecular identification was carried out at the Molecular Biology Laboratory, Medical Laboratory Technology, Faculty of Nursing and Health, University of Muhammadiyah Semarang. The 16S rRNA gene sequencing analysis was carried out at PT. Indonesian Genetics Science.

Isolation of bacteria culture from *C. racemosa*

Three grams of *C. racemosa* were put into a clean and dry plastic to be mashed. Then one spoon of *C. racemosa* was put into 5 ml of Physiological NaCl, homogenized. The suspension of *C. racemosa* was isolated by culturing it on NA and then incubated at 37°C for 24 hours. Then the bacteria were isolated again on BAP at 37°C for 24 hours. Hemolysis formed on BAP was observed against a bright light background. Colonies on BAP with a whole clear zone and clear green zone were inoculated on HIA (Heart Infusion Agar) fertilizing medium for 24 hours at 37°C in the Incubator. Colonies on HIA (Heart Infusion Agar) were cultured on BHI (Brain Heart Infusion) for 48 hours at 37°C in an incubator.

Isolation of DNA from *C. racemosa* bacterial culture

Bacterial colonies of *C. racemosa* culture from HIA were instilled in 5 ml of liquid BHI, then incubated at 37°C for 24 hours. The incubation results were centrifuged at 12000 rpm for 10 minutes at 4°C. The supernatant was discarded, then the pellet was added with 750 µl of lysis buffer, vortexed for a few seconds. 20 µl of proteinase K was added, shaken for 15 minutes using a shaker, then incubated at 55°C for 30 minutes, then centrifuged for 10 minutes at 12000 rpm at 4°C.

The supernatant solution was transferred to a 1.5 ml Eppendorf tube, and 700 µl of phenol CIAA was added, stirred slowly for 30 minutes, then centrifuged at 12000 rpm for 10 minutes at 4°C. The topmost part (aqueous phase) was transferred to an Eppendorf tube, then 96% ethanol was added in a 1:1 ratio, mixed gently until fine threads were seen, then centrifuged at 12000 rpm for 10 minutes at 4°C. The supernatant solution was discarded, the pellet was washed with ethanol 70%, then centrifuged at 12000 rpm for 10 minutes at 4°C.

The supernatant was discarded, then allowed to dry, then 100 µl of TE was added to dissolve the bacterial DNA to be seen on 1% agarose gel electrophoresis. The results of DNA isolation were pipetted as much as 2 µl, and the absorbance was measured using a UV nanodrop spectrophotometer at a wavelength of 260/280 nm. DNA purity has a ratio limit between 1.8-2.0. Obtained results of absorbance and concentration of DNA purity.⁶

Amplification of *C. racemosa* cultured bacteria

Nuclease Free Water was added as much as 7.5 µl and added 12.5 µl of taq polymerase enzyme into a PCR microtube. The forward and reverse primers were added 2 µl each, and 1 µl DNA samples were added to the microtube. The PCR temperature is set at the initial stage of denaturation (pre-denaturation) with 95°C for 6 minutes, followed by a denaturation stage at 95°C for 30 seconds, then annealing stage at 55°C for 30 seconds, extension at 72°C for 2 minutes.

The final extension stage was carried out at 72°C for 10 minutes and the cooling down the stage at 4°C for 6 minutes. The results of PCR amplification and markers were read using 2% agarose gel electrophoresis. PCR product approximately 1500 bp.

Data analysis

The purified 16S rRNA gene PCR product then proceeds to the sequencing stage to determine the sequence of nucleotide bases in the PCR product. The PCR product is sent to PT Genetics Science Indonesia for further processing to the sequencing analysis stage. The nucleotide sequence obtained is then used to compare the sequence data available at Genbank through the BLAST (Basic Local Alignment Search Tool) program at (NCBI), <https://blast.ncbi.nlm.nih.gov/>. The results of the sequencing were aligned and made a phylogenetic tree using Mega X.

RESULTS AND DISCUSSION

The bacterial culture of *C.racemosa* obtained four bacterial colonies. Bacterial colonies were grown on BAP media and produced a partial erythrocyte lysis zone. 4 Colonies of isolates of *C. racemose* had α -hemolysis type on BAP media ([Figure 1](#)).

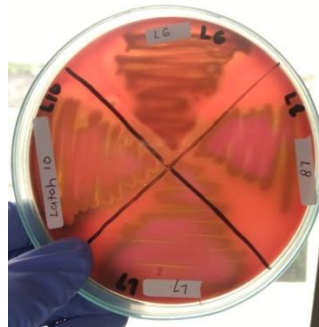


Figure 1. Characteristics of 4 bacterial colonies isolate *C.racemosa* in BAP

Four colonies of isolates of *C. racemosa* (CR1-AIK, CR6-AIK, CR7-AIK, and CR8-AIK) were pathogenic in humans because they were able to lyse some erythrocytes in BAP media. Each bacterial colony was purified, and Gram stain was performed to determine gram properties, morphology and confirm purity. CR1-AIK and CR6-AIK are Gram-positive bacilli. CR7-AIK is a slight rod-shaped gram-negative.

Each isolated bacterial colony of *C.racemosa* (CR1-AIK, CR6-AIK, CR7-AIK, and CR8-AIK) that had been purified was cultured on BHI media, and DNA isolation was performed using the phenol-CIAA method. The results of DNA isolation were used as a template for amplification of the 16S rRNA gene.

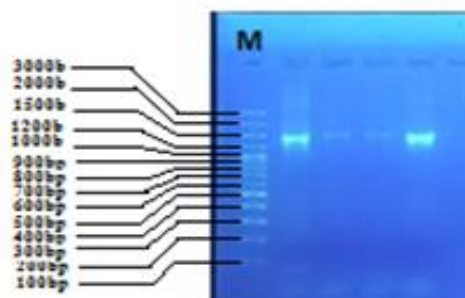


Figure 2. PCR product of about 1500 bp of *C.racemosa* isolate bacteria (CR1-AIK, CR6-AIK, CR7-AIK, and CR8-AIK).

Bacterial isolates CR1-AIK, CR6-AIK, CR7-AIK, and CR8-AIK had PCR products of about 1500 bp. The DNA band length of 1500 bp is specific to the target gene, namely 16S rRNA. In addition, the results of electrophoresis of PCR isolates of bacteria CR1-AIK, CR6-AIK, CR7-AIK, and CR8-AIK formed a single band (Figure 2). The PCR product of the 16S rRNA gene on bacterial isolates CR1-AIK, CR6-AIK, and CR7-AIK was then sequenced.

C. racemosa is processed without a cooking process, so that it is feared that there will be contaminant bacteria that can harm consumers. The habitat of *C. racemosa*, which is at the bottom of the waters, causes all parts of the harvested algae to contain sand, even sea pebbles. *C. racemosa* has abundant epibiotic bacteria and diatoms that adhere to the outer surface and form biofilms.⁷ Physical defenses such as mucus production in *C. racemosa* and diverse aquatic environments can form bacterial communities on the algal surface. Research related to bacteria from the surface of *C. racemosa* found no antimicrobial effect. Bacteria that live on the surface of *C. racemosa* form biofilms and produce secondary metabolites similar to algae.⁸

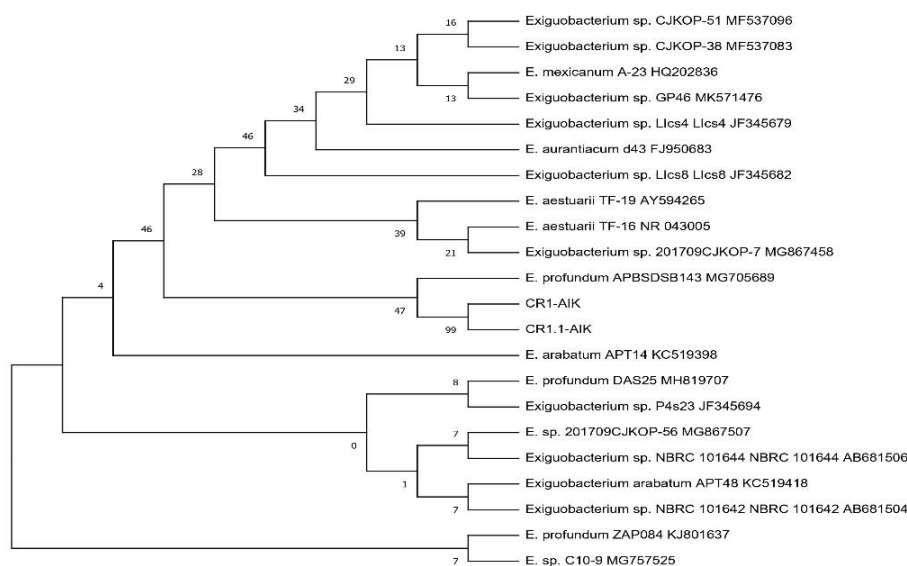


Figure 3. Phylogenetic analysis of the bacterial isolate CR1-AIK has a close similarity to *E. profundum*

Based on tree phylogenetic analysis using Mega X, the bacterial isolate CR1-AIK had similarities with the bacterium *Exiguobacterium profundum* (Figure 3). *E. profundum* has gene sequences with high similarity to the bacterial isolate CR1-AIK found from the Bay of Bengal Visakhapatnam Coast. Bacteria, firmicutes, bacilli, bacillales, *Exiguobacterium*. One of the bacteria found in *C. racemosa* is *Exiguobacterium profundum*.

Exiguobacterium profundum is a thermophilic bacteria that can produce lactic acid isolated from deep-sea hydrothermal. *E. profundum* is an anaerobic, halotolerant, moderately thermophilic bacterium and a bacterium that does not produce spores. *E. profundum* was isolated from the sea under hydrothermal conditions with an average depth of 2600m. *E. profundum* is a gram-positive bacterium, motile rod-shaped with peritrichous type and lives optimally at 45 °C, pH 7.2% NaCl. (+)-L-Lactate is the primary organic acid detected from carbohydrate fermentation by producing formate, acetate, and ethanol

compounds. Colonies formed circular, have a creamy or orange color. The characteristics of *E. profundum* are chemoorganotrophic and facultatively anaerobic. Biochemical tests showed that *E. profundum* had positive catalase and negative oxidase abilities. The substrates used for growth under anaerobic conditions were aesculin, amygdalin, arbutin, cellobiose, D-fructose, D-galactose, N-acetyl-D-glucosamine, D-glucose, gentiobiose, maltose, D-mannitol, D-mannose, D-ribose, salicin, starch, sucrose, and trehalose.⁹ The genus *Exiguobacterium* is not only found in *C. racemosa* but also red algae. *Exiguobacterium aestuarii* has been isolated from red algae, *Gracillaria corcata*. *E. aestuarii* produces magnesium ammonium phosphate (struvite) and can lyse agar (agarolytic). *E. aestuarii* can produce struvite, a bioremediation-based fertilizer on industrial phosphate waste.¹⁰ The discovery of the genus *Exiguobacterium* in the aquatic environment has the potential as a bioagent to control the explosion of the cyanobacterial population, namely *Microcystis aeruginosa*.¹¹

The genus *Exiguobacterium* is also found in marine organisms such as shrimp and fish. *Exiguobacterium arabatum* isolated from the stomach of shrimp (*Penaeus vannamei*) has potential as a probiotic because it has no resistance. Gene and only one virulence gene.¹² In addition, *Exiguobacterium arabatum* was also found in fish.¹³

Several genera of *Exiguobacterium* cause disease in humans. *Exiguobacterium* was found in periodontitis, myeloma, and infectious endocarditis patients, all of which isolates formed orange-yellow colonies on blood agar.¹⁴ Phylum Firmicutes species *Exiguobacterium* were also found in dishwashing water that had formed biofilms. Several species in this genus are known for their ability to proliferate in extreme environments such as hot, alkaline, and marine environments.¹⁵ The genus *Exiguobacterium* causes pathogens in humans because it has virulence factors including tlyC, MprR, MCP, Dam, which play an essential role in lethal infections.¹⁶

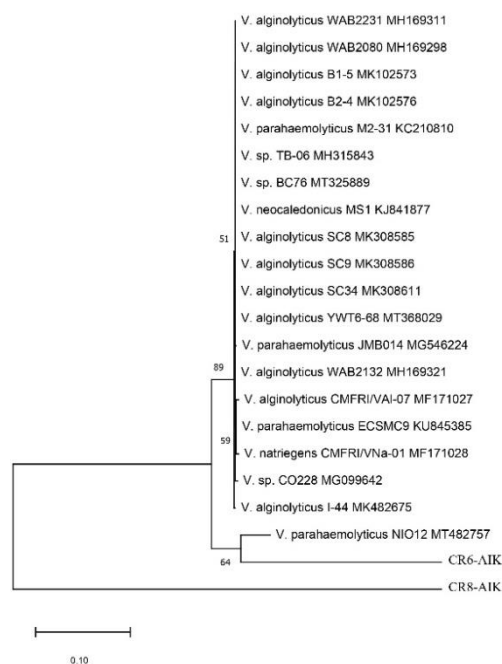


Figure 4. phylogenetic analysis of bacterial isolate CR6-AIK has a close similarity to *V. parahaemolyticus*

The results of the phylogenetic tree analysis using Mega X showed that the bacterial isolate CR6-AIK had a close relationship with *Vibrio parahaemolyticus* found in aquatic areas (figure 4). *Vibrio parahaemolyticus* is a bacteria, proteobacteria, gammaproteobacteria, vibriionales, Vibrionaceae, and the genus Vibrio.

The genus Vibrio is a bacterium that is mainly found in marine environments. Vibrios develop in the bodies of marine animals. Vibrios that live in the bodies of marine animals are mutualism, for example, *Vibrio fischeri*-squid. Vibrio interactions with the host are also parasitic and pathogenic to humans, for example, *Vibrio harveyi*-shrimp. The genus Vibrio is vast and is divided into several species groups that have different ecological environments. Vibrio is not only found in *C. racemosa*, but also many other marine organisms. For example, *V. harveyi* is a species associated with disease-causing various marine animals. *Vibrio coralliilyticus* is involved in bleaching events on corals. *V. splendidus* a group of species involved with a disease in mollusks, *V. haliotocoli* is a species that have the potential to have mutualistic relationships with abalones organisms and act as probionts. *V. cholera* is a type of vibrio pathogen in humans.^{11,17}

CR6-AIK is closely related to *Vibrio parahaemolyticus*. *Vibrio parahaemolyticus* is naturally found in aquatic areas and is associated with gastroenteritis either from contaminated raw food or undercooked seafood^{18,19} and is life-threatening in immunocompromised patients. *V. parahaemolyticus* is a halophilic Gram-negative bacterium. Several *V. parahaemolyticus* were identified to cause opportunistic diseases in humans, causing subacute gastroenteritis with dehydration, fever, and of various ages, as well as gender.²⁰ *V. parahaemolyticus* causes foodborne disease. *V. parahaemolyticus* has different genetic distribution, genetic elements, and pathogenicity depending on the environment.²¹ *V. parahaemolyticus* isolated from 3 main water areas in China has different genetic diversity and population structures.^{20,22} Based on the analysis using WGS, there were differences in the pathogenicity of *V. parahaemolyticus* in various aquatic environments resulting from the combination of horizontal genome transfer, pathological distribution elements.^{11,13,18} *V. parahaemolyticus* was also found to cause death in shrimp due to acute hepatopancreatic necrosis disease.²³

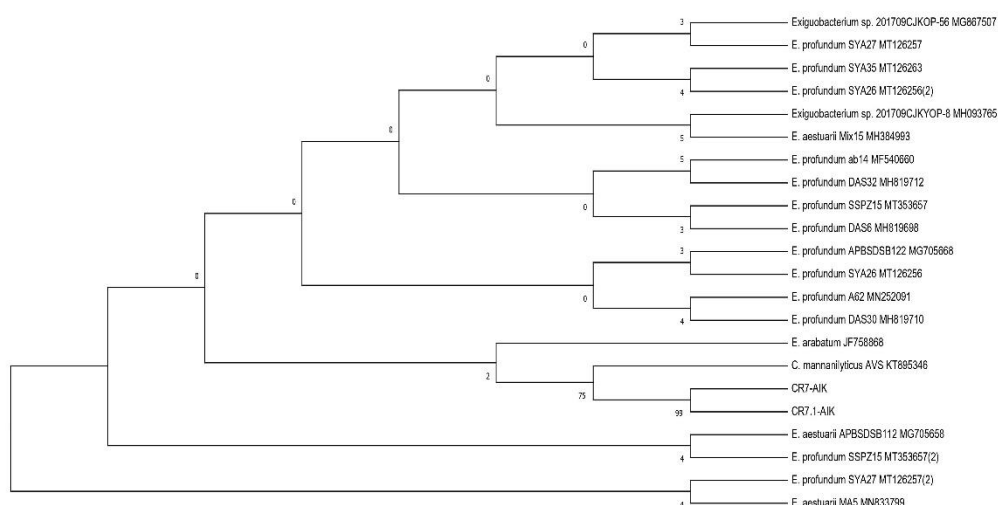


Figure 5. Phylogenetic analysis of the bacterial isolate CR7-AIK has a close similarity to *Caldalkalibacillus mannanilyticus*

Based on phylogenetic analysis using Mega X Isolate, CR7-AIK bacteria have a relationship with *Caldalkalibacillus mannanilyticus* (Figure 5). *C. mannanilyticus* has been found in textile factory wastewaters.²³ *C. mannanilyticus*, bacteria, firmicutes, bacilli, bacillales, bacillaceae, caldalkalibacillus. Besides being found in *C. racemose*, *C. mannanilyticus* were found on the bottom of the water in hypersaline aiding lake conditions. One of the alkaliphilic bacteria that can be used for industrial enzyme applications is *Bacillus mannanilyticus* sp. *Bacillus mannanilyticus* is capable of producing alkaline enzymes such as proteases. *Caldalkalibacillus thermarum* produces extracellular enzymes that are resistant to high pH and high-temperature conditions. Aerobic, endospore-forming, obligate alkaliphilic.^{24,25,26}

CONCLUSION

The bacterial isolate CR1-AIK had a high similarity with the bacterium *Exiguobacterium profundum*, commonly found in marine areas. Isolate bacteria CR6-AIK has similarities with bacteria *Vibrio parahaemolyticus*, which can cause foodborne disease. Genus *Exiguobacterium* and *Vibrio* can cause infectious diseases in humans. CR7-AIK has a similarity with the bacteria *Caldalkalibacillus mannanilyticus* and is widely used in the enzyme industry. Consumption of fresh *C.racemosa* must go through a clean wash because the algae contain pathogenic bacteria that cause foodborne disease.

AUTHORS' CONTRIBUTIONS

Aprilia Indra Kartika : Analysing sequencing data, compiling publication manuscript
Meutia Srikandi Fitria : Bacteria culture, NA, BAP, BHI
Vanny Oktaviola : DNA extraction, PCR
All authors contributed equally to this work.

FOUNDING INFORMATION

This research received DOSEN PRATAMA Grant from LPPM Universitas Muhammadiyah Semarang, Indonesia.

DATA AVAILABILITY STATEMENT

The utilized data to contribute to this investigation are available from the corresponding author on reasonable request. Sequencing data in FASTA format, bacteria culture from NA, BHI, and BAP is very open to be accessed by anyone.

DISCLOSURE STATEMENT

The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors. The data is the result of the author's research and has never been published in other journals

REFERENCE

1. Wantania LL, Ginting EL, Wullur S. Isolasi Bakteri Symbion dengan Spons dari Perairan Tongkeina, Sulawesi Utara. *J LPPM Bid SAINS DAN Teknol.* 2016;3(1):57-65.
<https://ejournal.unsrat.ac.id/index.php/lppmsains/article/view/15208>.
2. Sri Rahmaningsih, Sri Wilis AM. Bakteri Patogen dari Perairan Pantai dan Kawasan Tambak di Kecamatan Jenu Kabupaten Tuban. *Ekol J Ilm Ilmu Dasar dan Lingkung Hidup.* 2012;12(1):1-5. [doi:10.33751/ekol.v12i1.248](https://doi.org/10.33751/ekol.v12i1.248).
3. Russell FM, Biribo SSN, Selvaraj G, et al. As a Bacterial Culture Medium, Citrated Sheep Blood Agar Is a Practical Alternative to Citrated Human Blood Agar in Laboratories of Developing Countries. *J Clin Microbiol.*

- 2006;44(9):3346-3351. [doi:10.1128/JCM.02631-05](https://doi.org/10.1128/JCM.02631-05).
4. Radji M, Puspaningrum A, Sumiati A. Deteksi Cepat Bakteri Escherichia coli Dalam Sampel Air Dengan Metode Polymerase Chain Reaction Menggunakan Primer 16E1 dan 16E2. *MAKARA Sci Ser.* 2011;14(1):39-43. [doi:10.7454/mss.v14i1.474](https://doi.org/10.7454/mss.v14i1.474).
 5. Singh V, Chaudhary D, Mani I. Molecular characterization and modeling of secondary structure of 16S rRNA from Aeromonas veronii National Bureau of Fish Genetic Resources , Canal Ring Road , PO- Dilkusha , Lucknow ,. *Int J Appl Biol Pharm Technol.* 2012;3(1). <https://www.semanticscholar.org/paper/MOLECULAR-CHARACTERIZATION-AND-MODELING-OF-OF-16S-Singh-Chaudhary/8ee2c44ee39ce36a22b162c612eee6db0c8939bf>.
 6. Chen H, Rangasamy M, Tan SY, Wang H, Siegfried BD. Evaluation of Five Methods for Total DNA Extraction from Western Corn Rootworm Beetles. Lalueza-Fox C, ed. *PLoS One.* 2010;5(8):e11963. [doi:10.1371/journal.pone.0011963](https://doi.org/10.1371/journal.pone.0011963).
 7. Aires T, Serrão EA, Kendrick G, Duarte CM, Arnaud-Haond S. Invasion Is a Community Affair: Clandestine Followers in the Bacterial Community Associated to Green Algae, Caulerpa racemosa, Track the Invasion Source. Verbruggen H, ed. *PLoS One.* 2013;8(7):e68429. [doi:10.1371/journal.pone.0068429](https://doi.org/10.1371/journal.pone.0068429).
 8. Dobretsov S, Dahms H, Harder T, Qian P. Allelochemical defense against epibiosis in the macroalga Caulerpa racemosa var. turbinata. *Mar Ecol Prog Ser.* 2006;318:165-175. [doi:10.3354/meps318165](https://doi.org/10.3354/meps318165)
 9. Crapart S, Fardeau M, Cayol J, et al. Exiguobacterium profundum sp. nov., a moderately thermophilic, lactic acid-producing bacterium isolated from a deep-sea hydrothermal vent. *Int J Syst Evol Microbiol.* 2007;57(2):287-292. [doi:10.1099/ijs.0.64639-0](https://doi.org/10.1099/ijs.0.64639-0).
 10. Leela S, Ranishree J, Perumal R, Ramasamy R. Characterization of Struvite Produced by an Algal Associated Agarolytic Bacterium Exiguobacterium aestuarii St. SR 101. *J Pure Appl Microbiol.* 2019;13(2):1227-1234. [doi:10.22207/JPAM.13.2.64](https://doi.org/10.22207/JPAM.13.2.64)
 11. Li Y, Liu L, Xu Y, et al. Stress of algicidal substances from a bacterium Exiguobacterium sp. h10 on Microcystis aeruginosa. *Lett Appl Microbiol.* 2017;64(1):57-65. [doi:10.1111/lam.12678](https://doi.org/10.1111/lam.12678).
 12. Cong M, Jiang Q, Xu X, Huang L, Su Y, Yan Q. The complete genome sequence of Exiguobacterium arabatum W-01 reveals potential probiotic functions. *Microbiologyopen.* 2017;6(5):e00496. [doi:10.1002/mbo3.496](https://doi.org/10.1002/mbo3.496).
 13. Yang J, Wang C, Wu J, Liu L, Zhang G, Feng J. Characterization of a Multiresistant Mosaic Plasmid from a Fish Farm Sediment Exiguobacterium sp. Isolate Reveals Aggregation of Functional Clinic-Associated Antibiotic Resistance Genes. *Appl Environ Microbiol.* 2014;80(4):1482-1488. [doi:10.1128/AEM.03257-13](https://doi.org/10.1128/AEM.03257-13).
 14. Pitt TL, Malnick H, Shah J, et al. Characterisation of Exiguobacterium aurantiacum isolates from blood cultures of six patients. *Clin Microbiol Infect.* 2007;13(9):946-948. [doi:10.1111/j.1469-0691.2007.01779.x](https://doi.org/10.1111/j.1469-0691.2007.01779.x).
 15. Shatila F, Yusef H, Holail H. Original Research Article Pigment production by Exiguobacterium aurantiacum FH , a novel Lebanese strain. *Int J Curr Microbiol Appl Sci.* 2013;2(12):176-191.
 16. Chen X, Wang L, Zhou J, et al. Exiguobacterium sp. A1b/GX59 isolated from a patient with community-acquired pneumonia and bacteremia: genomic characterization and literature review. *BMC Infect Dis.* 2017;17(1):508. [doi:10.1186/s12879-017-2616-1](https://doi.org/10.1186/s12879-017-2616-1).
 17. Thompson CC, Thompson FL, Vicente ACP, Swings J. Phylogenetic analysis of vibrios and related species by means of atpA gene sequences. *Int J Syst Evol Microbiol.* 2007;57(11):2480-2484. [doi:10.1099/ijs.0.65223-](https://doi.org/10.1099/ijs.0.65223-)

- Q.
18. Liu J, Qin K, Wu C, Fu K, Yu X, Zhou L. De Novo Sequencing Provides Insights Into the Pathogenicity of Foodborne *Vibrio parahaemolyticus*. *Front Cell Infect Microbiol.* 2021;11(May):1-21. [doi:10.3389/fcimb.2021.652957](https://doi.org/10.3389/fcimb.2021.652957).
 19. Urmsersbach S, Alter T, Koralage MSG, et al. Population analysis of *Vibrio parahaemolyticus* originating from different geographical regions demonstrates a high genetic diversity. *BMC Microbiol.* 2014;14:59. [doi:10.1186/1471-2180-14-59](https://doi.org/10.1186/1471-2180-14-59).
 20. Martinez-Urtaza J, Trinanés J, Abanto M, et al. Epidemic Dynamics of *Vibrio parahaemolyticus* Illness in a Hotspot of Disease Emergence, Galicia, Spain. *Emerg Infect Dis.* 2018;24(5):852-859. [doi:10.3201/eid2405.171700](https://doi.org/10.3201/eid2405.171700).
 21. Montieri S, Suffredini E, Ciccozzi M, Croci L. Phylogenetic and evolutionary analysis of *Vibrio parahaemolyticus* and *Vibrio alginolyticus* isolates based on *toxR* gene sequence. *New Microbiol.* 2010;33(4):359-372. <http://www.ncbi.nlm.nih.gov/pubmed/21213595>.
 22. Li P, Xin W, Kang L, et al. Genetic and population analyses of *Vibrio parahaemolyticus* isolates from three major coastal regions in China. *Future Microbiol.* 2018;13:1261-1269. [doi:10.2217/fmb-2018-0060](https://doi.org/10.2217/fmb-2018-0060).
 23. Kumar R, Tung T-C, Ng TH, et al. Metabolic Alterations in Shrimp Stomach During Acute Hepatopancreatic Necrosis Disease and Effects of Taurocholate on *Vibrio parahaemolyticus*. *Front Microbiol.* 2021;12(April). [doi:10.3389/fmicb.2021.631468](https://doi.org/10.3389/fmicb.2021.631468).
 24. Nogi Y, Takami H, Horikoshi K. Characterization of alkaliphilic *Bacillus* strains used in industry: proposal of five novel species. *Int J Syst Evol Microbiol.* 2005;55(6):2309-2315. [doi:10.1099/ijs.0.63649-0](https://doi.org/10.1099/ijs.0.63649-0).
 25. de Jong SI, van den Broek MA, Merkel AY, et al. Genomic analysis of *Caldalkalibacillus thermarum* TA2.A1 reveals aerobic alkaliphilic metabolism and evolutionary hallmarks linking alkaliphilic bacteria and plant life. *Extremophiles.* 2020;24(6):923-935. [doi:10.1007/s00792-020-01205-w](https://doi.org/10.1007/s00792-020-01205-w).
 26. Xue Y, Zhang X, Zhou C, et al. *Caldalkalibacillus thermarum* gen. nov., sp. nov., a novel alkalithermophilic bacterium from a hot spring in China. *Int J Syst Evol Microbiol.* 2006;56(6):1217-1221. [doi:10.1099/ijs.0.64105-0](https://doi.org/10.1099/ijs.0.64105-0).