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研究課題名（和文）How did marine bacterium Vibrio cholerae adapted to freshwater environment?

研究課題名（英文）How did marine bacterium Vibrio cholerae adapted to freshwater environment?

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研究成果の概要（和文）：本研究は、病原性海洋ビブリオ細菌の淡水環境への適応を解明することを目的とした。155株を収集し、暫定的な同定を行った。その結果、Vibrio cholerae、V. vulnificus、V. parahaemolyticusと同定された菌株は、全ゲノム配列が決定された。その選択された菌株について、非接触型濁度計ODboxを用いて、0 %、0.2 %、1 %のNaCl環境下での増殖能力を分析した。異なるNaCl濃度における種特異的な増殖パターンが記録された。現在、3種の淡水への適応に関わる遺伝子の同定を目的とした解析を行っている。

研究成果の学術的意義や社会的意義
The results of the research will help to elucidate how marine bacteria adapt to the freshwater environment. The freshwater environment has different chemical parameters from the marine environment, yet marine pathogenic bacteria can survive, reproduce and infect humans in freshwater.

研究成果の概要（英文）：This study aimed at understanding pathogenic marine Vibrio bacteria adaptation to freshwater environments. We collected 155 strains and performed a provisional identification. Selected strains identified as Vibrio cholerae, V. vulnificus, and V. parahaemolyticus had their whole genomes sequenced. In addition, the selected strains were analyzed for their ability to grow in the environment with 0 %, 0.2 %, and 1 % NaCl using an ODbox, a non-contact turbidimeter. Species-specific growth patterns at different NaCl concentrations were documented. We are currently performing an analysis aimed at the identification of genes involved in the three species' adaptation to freshwater. The analysis relies on the whole genome sequence data and growth data from different NaCl environments for the identification of the freshwater adaptation genes.

研究分野：Microbiology

キーワード：Vibrio

様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９（共通）

１．研究開始当初の背景

Vibrio bacteria are some of the deadliest human pathogens, responsible for millions of infection cases and thousands of deaths every year. The bacteria are particularly dangerous in areas where natural disasters or armed conflict lead to pollution of drinking water by human feces or seawater. The majority of *Vibrio* species are native to marine environments, which is significantly different from freshwater in regards to salinity, pH, and redox conditions. Only a few *Vibrio* species have been reported to grow in freshwater or media containing 0% NaCl. One of these species is the deadly human pathogen *Vibrio cholerae*. Most *V. cholerae* strains are unable to grow in an environment without NaCl, but they can survive in low-salt concentration environments for several hours or days. However, recent studies found some *V. cholerae* strains which can grow (not just survive) in an environment with 0% NaCl.

Understanding *V. cholerae* adaptation to freshwater is important because the growth of the bacterium in drinking water increases the risk of cholera outbreaks. Most research on human pathogenic *Vibrio* focuses on clinical applications: pathogenicity, serotypes diversity, or evolution of pandemic variants. Only a few studies analyzed how the bacteria can survive and grow in freshwater. Recent ecological studies found an increase in the number of *Vibrio* species in coastal brackish and freshwater environments. An increase in the number of *Vibrio* in brackish and freshwater environments was linked to increasing global ocean temperatures. These new *Vibrio* colonizers come in contact with humans and animals, and could potentially cause sporadic outbreaks of diseases. However, it is still unclear how halophilic *Vibrio* species can adapt to growth in freshwater.

２．研究の目的

This project aimed to answer two questions:

Q1: How did *V. cholerae* adapt to freshwater?

This question is fundamental to our understanding of *V. cholerae* distribution in freshwater. Only some *V. cholerae* strains can grow in freshwater, while most strains are only able to survive (but not grow) in freshwater. There are two hypotheses explaining *V. cholerae* adaptation to freshwater:

- Horizontal transfer of genes allowing adaptation to freshwater. No evidence of the horizontal transfer or what genes could be involved in adaptation was identified so far.
- Some research suggested that a reduction in the chromosome number from two chromosomes to a single chromosome organization played a role in the adaptation to freshwater. This hypothesis was never thoroughly tested.

Q2: Why can’t most *Vibrio* species grow in freshwater and how could they adapt to the freshwater environment?

The answer to the second question is also of great importance. Among 146 described *Vibrio* species majority have never been found in freshwater or are unable to grow in media without NaCl. However, there is a possibility that given suitable environmental conditions marine *Vibrio* could colonize freshwater in coastal areas, similar to *V. cholerae*. The likelihood of the bacteria adaptation to freshwater is increased by changes

to the global environment, particularly increasing the temperature of marine and coastal environments. After adaptation to freshwater, these bacteria could emerge as new human pathogens. This study's results will help predict how these new *Vibrio* pathogens could emerge.

３．研究の方法

The study plan aimed to isolate *V. cholerae* from freshwater and seawater samples collected in the Miyazaki prefecture. The isolated bacteria would be provisionally identified using a simple MLSA scheme based on sequences of housekeeping genes. Selected strains would be tested for growth in media supplemented with different NaCl concentrations. The genomic organization of the selected *V. cholerae* strains would be analyzed using pulse-filed-gel-electrophoresis (PFGE). Also, the selected strains would have their whole genome sequence analyzed. The genome sequences of these strains would be analyzed for accurate taxonomic classification to identify *V. cholerae* and related pathogenic *Vibrio* species, and genome-wide association studies (GWAS) would also be conducted. Additionally, the study would use an experimental evolution approach to analyze how *V. cholerae* adapts to an environment with low NaCl concentration.

４．研究成果

(1) Isolation of bacteria strains.

We isolated 155 strains of *Vibrionaceae* from samples of freshwater collected from the Kaeda River in the Miyazaki prefecture. The sampling was done along the Kaeda River, near the estuary and upstream of the estuary mouth. A total of six sampling experiments were conducted during the study. Water parameters were recorded. Samples were screened for the presence of *Vibrionaceae* by using the TCBS medium, followed by further screening to select *Vibrio cholerae* using CHROMagar Vibrio medium. The genomic DNA of the isolated 155 strains was isolated and used as a template for PCR amplification of gene sequences used in an MLSA-based identification scheme.

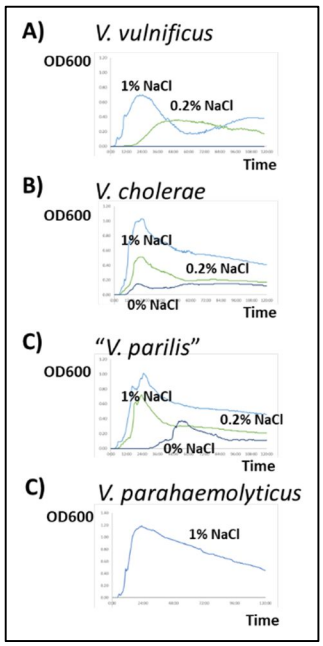
Overall, the selected screening method proved to be simple and efficient. However, among the six collected water samples, only three samples contained *V. cholerae*, for a total of 16 strains. However, in addition to *V. cholerae,* we isolated a large number of strains from other human pathogenic *Vibrio* species, namely *V. parahaemolyticus* and *V. vulnificus*. Due to an insufficient number of isolated *V. cholerae* strains for the proposed study, we decided to expand the scope of the study and analyze the adaptation of three human pathogenic *Vibrio* species to freshwater, namely *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus.*

(2) Characterization of the bacteria growth in an environment with low NaCl concentrations.

Initially, we decided to analyze the growth of 15 isolated *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus* strains (five from each species). The bacteria were grown on basal medium supplemented with 0%, 0.2%, 1%, or 2% NaCl. However, the results proved difficult to interpret. All the analyzed strains grew well on media containing 1% or 2% NaCl, but at lower NaCl concentrations we observed some variability. *V. parahaemolyticus* strains usually did not grow at 0% and 0.2%, but when repeating the experiment some strains sometimes showed weak or showed delayed growth after several days. Some

variance was also observed when analyzing *V. cholerae* strains.

The observed variance would make the following genome-wide association studies (GWAS) difficult and likely lead to inexact results. For good quality results, GWAS requires a clear separation between the analyzed genomic data, but the observed variability using basal medium supplemented with NaCl could not provide the required quality. As a result, we decided to make an improvement to the proposed research plan and analyze the bacteria growth using a non-contact turbidimeter. We purchased an ODBox-C monitor unit (TAITEC) which allows non-contact measurements of turbidity in a growing bacteria culture, over a long period. Compared to analyzing the bacteria growth on an agar-solidified medium, using ODBox we can obtain accurate information about the growth pattern of the bacteria over a long period. We used the newly purchased equipment to monitor the growth of 15 isolated *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus* strains in a liquid medium supplemented with 0%, 0.2%, and 1% NaCl. The bacteria were grown for five days at 28ºC. Growth on a nutrient-rich LSW-70 medium was used as a control. Each growth experiment was repeated three times for each of the 15 strains.



Results of the experiments revealed that all analyzed *V. vulnificus* strains grew on well basal medium supplemented with 1% NaCl (Figure 1A). Growth on basal medium supplemented with 0.2% NaCl was delayed, no growth was observed on basal medium supplemented with 0% NaCl. Four of the analyzed *V. cholerae* strains grew well on all analyzed basal media (Figure 1B). One strain showed a different growth pattern, with delayed growth on basal medium supplemented with 0% NaCl (Figure 1C). This strain was later taxonomically classified as "*V. parilis*" using a genomic analysis (see below). Analyzed *V. parahaemolyticus* strains usually did not grow on basal media supplemented with 0% or 0.2% NaCl (Figure 1D). However, one *V. parahaemolyticus* strain in some repeats of the experiment showed growth on basal medium supplemented with 0% or 0.2% NaCl.

(3) Genome sequencing and GWAS.

We obtained the whole genome sequences of seven *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus* strains. Currently, we are in the process of sequencing the genomes of the remaining 8 analyzed strains. Once the remaining genome sequences are complete, we will be able to continue with the genome-wide association studies (GWAS). The GWAS analysis is the last remaining part of the study.

We used the obtained genome sequences for accurate taxonomic classification. We calculated the average nucleotide identity (ANI) between the obtained genome sequences and the genome sequences of type strains of *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus*, as well as genome-based phylogenetic analyses. The analyses revealed that our MLSA scheme accurately identified all *V. parahaemolyticus* strains. One strain initially classified as *V. cholerae* was later reclassified as "*V. parilis*", which is a recently

discovered *Vibrio* species, whose name is not yet validly described using bacterial taxonomy rules. The "*V. parilis*" strain showed a different pattern of growth in basal medium supplemented with NaCl compared to *V.* cholerae strains (see above). One strain initially identified as *V. vulnificus* showed low ANI values compared to the type strain of the species, further taxonomic classification of the strain will be required. The strain has the same growth pattern as other analyzed *V. vulnificus*, which will have no impact on GWAS analysis.

The proposed plan called for an analysis of the genome organization of the isolated strains using the PFGE. However, this analysis is not necessary thanks to the high quality of the genome sequencing results. The obtained genome sequences could be assembled into complete chromosomes and revealed no obvious changes to the chromosomal organization compared to other *Vibrionaceae*. We can therefore reject a hypothesis that changes in genome organization had an impact on the bacteria's ability to grow in a low NaCl environment.

(4) Experimental evolution.

The proposed plan called for an experimental evolution approach to analyze *V. cholerae* adaptation to freshwater. We attempted the planned experiments but got very mixed results. The media we used allowed delayed growth of *V. cholerae* strains meaning that the strains were already adapted to the conditions we used. However, thanks to the data obtained from the non-contact measurements of turbidity in cultures grown in basal medium with different NaCl concentrations, we decided to change the experimental evolution approach. Instead of *V. cholerae*, we plan to conduct the experimental evolution with *V. parahaemolyticus* strains.

５．主な発表論文等

〔雑誌論文〕　計0件

〔学会発表〕　計0件

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関