様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

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研究課題名（和文）Structural and functional studies of iron uptake ATP-binding cassette transporters (ABC transporters) in Gram-negative bacteria

研究課題名（英文）Structural and functional studies of iron uptake ATP-binding cassette transporters (ABC transporters) in Gram-negative bacteria

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研究成果の概要（和文）：ビブリオ菌Vibrio. metschnikovii由来のPBP（FbpA）の鉄結合を選択的に阻害する食品成分を香辛料・ハーブの熱水抽出物から探索し、ロスマリン酸(RA)という化合物を同定できた。鉄取り込み阻害剤として、RAの抗菌活性がクエン酸ナトリウム共存下で増強されることを見出した。さらに、V. metschnikovii由来のFbpAの鉄結合部位にRAが結合した結晶構造も解いて、鉄取込みを阻害する分子機構を明らかにした。なお、ビブリオ感染症の原因菌であるV. parahaemolyticus、V. alginolyticus、V. vulnificusに対しても効果が示した。

研究成果の学術的意義や社会的意義
学術的意義：ビブリオ菌由来の鉄結合タンパク質FbpAの構造解析によって、菌のFe3+取り込み機構を明らかにした。更に、FbpAを阻害する機構を持つロスマリン酸（RA）との複合体の構造解析により、その分子機構も可視化できた。
社会的意義：本研究から得られるビブリオ属細菌に対する静菌剤は、ヒトにとって安全な食品成分からなり、抗菌スペクトルが狭く選択的な静菌作用を示した。食品生産、保管、流通における使用だけでなく、消費者も安心して利用可能である。畜産・水産・植物栽培、ヒト医療・獣医医療における抗生物質の過剰・不適切な使用を減らすことに貢献する。

研究成果の概要（英文）：Food ingredients screening from hot water extracts of spices and herbs that specifically inhibit iron binding of PBP (FbpA) from Vibrio. metschnikovii were performed and a compound, rosmarinic acid (RA), from rosemary extracts was identified as an inhibitor of iron uptake. The bacteriostatic activity of RA was confirmed and can be enhanced in the presence of sodium citrate (SC). The crystal structure of RA bound to the iron-binding site of FbpA from V. metschnikovii was solved and the mechanism of iron uptake inhibition was revealed at the molecular level. Moreover, bacteriostatic activity of RA and SC can also be overserved in other pathogenic vibrio species (e.g. V. parahaemolyticus, V. alginolyticus, and V. vulnificus).

研究分野：農芸化学

キーワード：FbpA　vibriosis　spice extracts　rosmarinic acid　Vibrio species　bacteriostatic agent

様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９（共通）

１．研究開始当初の背景

Bacterial resistance to most of the antibiotics is rapidly occurring worldwide. The challenge of modern medicine is to discover new strategies to combat multidrug-resistant bacteria, especially Gram-negative bacteria for which the situation is more critical.

V. metschnikovii is a model bacterium amongst the marine pathogenic *Vibrio* species (e.g. *V. vulnificus*, *V. parahaemolyticus*, and *V. cholerae*), which contaminates seafood and causes human illness such as vibriosis. Although the cases of vibriosis is not frequently reported in Japan, approximately 80,000 cases of vibriosis were reported each year in the United States, and the situation is getting more serious due to global warming.

Iron is an essential nutrient for both animals and pathogenic bacteria. Bacteria compete the hosts for iron absorption during infection. Thus, the restriction of iron uptake has become an alternative way to inhibit the survival of bacteria. FbpBC/A is a unique ATP-binding cassette (ABC) importer for Fe^{3+} uptake that exclusively exists in Gram-negative bacteria. This project is to 1) study function and structure of FbpBC/A; 2) inhibit FbpBC/A to impair the ability of Fe^{3+} uptake and the growth of Gram-neg ative bacteria.

２．研究の目的

1) To reveal the Fe^{3+}-binding and release mechanisms of FbpA.
2) To determine the structures of FbpBC and FbpBC-A complexes.
3) To inhibit the growth of *Vibrio* species, based on iron restriction via the inhibition of FbpA.

３．研究の方法

1) To reveal the Fe^{3+}-binding and release mechanisms of FbpA.
FbpA from *V. metschnikovii* (Vm) was overexpressed in *Escherichia coli* and purified. The Fe^{3+}-binding and release mechanisms of VmFbpA was analyzed and compared by gel filtration chromatography and spectroscopy.
2) To determine the structures of FbpBC and FbpBC-A complexes.
FbpA and FbpBC complex from a model bacterium, *T. thermophilus* HB8 (Tt), were overexpressed in *E. coli* and purified. The structure of TtFbpBC complex was analyzed by X-ray crystallography and cryogenic electron microscopy (cryo-EM). TtFbpBC-A complex was be prepared by pull-down assay and the structure was analyzed by cryo-EM. The factors that control Fe^{3+} release from TtFbpA to TtFbpB was elucidated by pull-down assay.
3) To inhibit the growth of *Vibrio* species, based on iron restriction via the inhibition of FbpA.
The screening of Fe^{3+}-VmFbpA interaction inhibitors was performed using the water extracts of 20 different spices. The specific agents that inhibit the Fe^{3+} binding of VmFbpA and the growth of *V. metschnikovii* was determined and analyzed. The best dosage of the antibacterial agents that inhibit the growth of *V. metschnikovii* was optimized and their antimicrobial spectrum against other Vibrio species. (e.g. *V. vulnificus* and *V. parahaemolyticus*) was investigated.

４．研究成果

1) To reveal the Fe^{3+}-binding and release mechanisms of FbpA.
VmFbpA was successfully expressed and purified at 30° C induced by 0.001 mM IPTG (**Figure 1 AB**). When VmFbpA was incubated by excessive EDTA or FeCl3, only one peak was observed in each gel filtration chromatography. The elution volume of Fe^{3+}-VmFbpA (15.1 mL) was higher than that of apo VmFbpA (13.8 mL) (**Figure 1 CD**), which demonstrated that VmFbpA becomes more compact when Fe^{3+} bound to it. Besides, the UV-Vis (300-700 nm) spectra showed that Fe^{3+}-VmFbpA had a specific absorbance at 412 nm but such absorbance was not observed in apo VmFbpA.

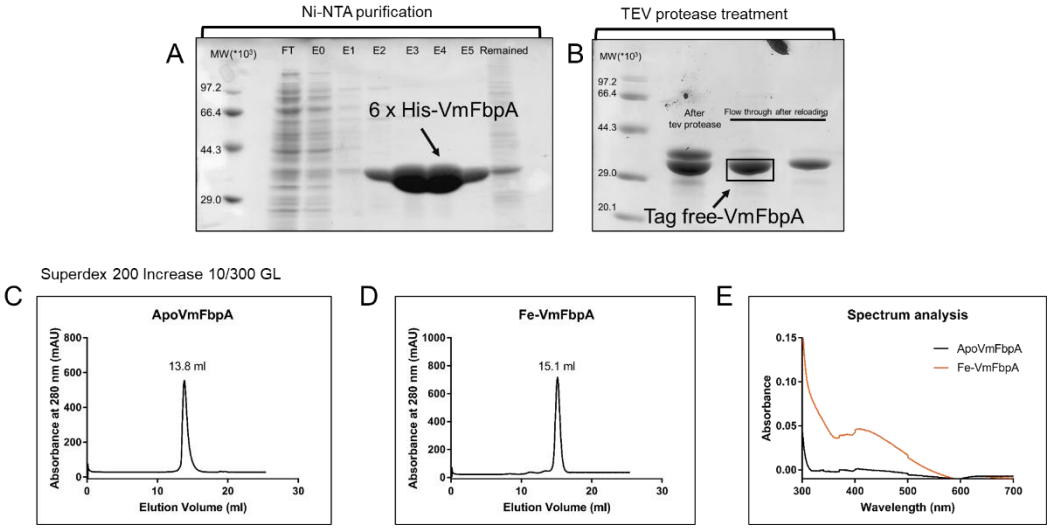

Figure 1. Purification and analysis of Fe^{3+} bound VmFbpA and apo VmFbpA. **A**: SDS-PAGE for the fractions after Ni^{2+} resin affinity chromatography. **B**: SDS-PAGE for the verification of 6 × His-tag cleavage. **C**: Gel filtration result of apo VmFbpA. **D**: Gelfiltration result of Fe^{3+}-VmFbpA. **E**: Absorbance spectrum (300-700 nm) of Fe3+-VmFbpA and Apo VmFbpA.

2) To determine the structures of FbpBC and FbpBC-A complexes.

To solve the structures of TtFbpBC and TtFbpBC-A complexes, X-ray crystallograpic and cryo-EM analyses were performed. TtFbpBC was successfully overexpressed in Escherichia coli BL21(DE3), solubilized with 2.0% DDM, and purified by immobilized Ni^{2+} affinity chromatography and gel filtration chromatography (**Figure 2**). The pull-down assay showed that apo-TtFbpA binds stronger to the TtFbpBC complex than Fe^{3+}-TtFbpA . The assay data indicated that the presence of Mg^{2+} is much more important than the type of nucleotides in stabilizing TtFbpBC-A complex (**Figure 3**). Crystallization of TtFbpBC and TtFbpBC-A complexes were performed by vapour diffusion and lipidic cubic phase (LCP) methods using a variety of reservoir but only TtFbpBC formed crystals. The best TtFbpBC crystals were formed in the presence of 0.2% n-fecyl-β-D-maltopyranoside (DM) and 20% PEG2000, which diffracted X-rays to ~4.0 Å (**Figure 4**).

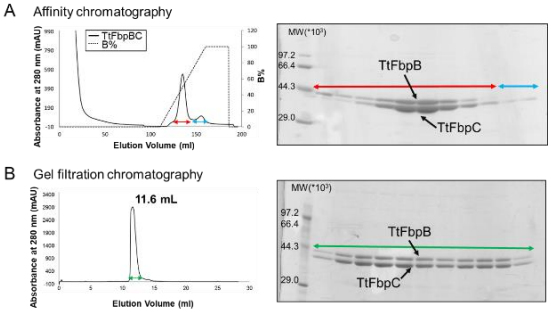

Figure 2. Expression and purification of TtFbpBC. **A**: Immobulized Ni^{2+} affinity chromatography and purity check by SDS-PAGE. **B**: Gel filtration chromatography and purity check by SDS-PAGE.

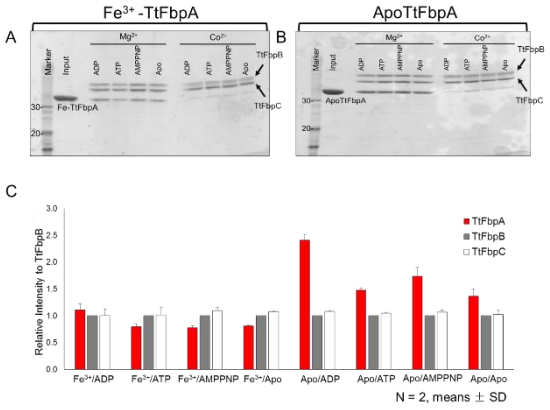

Figure 3. His-tag pull-down assay between TtFbpA and TtFbpBC. **A**: The effect of nucleotides (ADP, ATP, AMPPNP) and divalent metal (Mg^{2+} or Co^{2+}) was assessed in the presence of Fe^{3+}. **B**: The effect of nucleotides (ADP, ATP, AMPPNP) and divalent metal (Mg^{2+} or Co^{2+}) was assessed in the absence of Fe^{3+}. **C**: The relative intensity of each band obtained in A and B was measured by Image-J and normalized by TtFbpB.

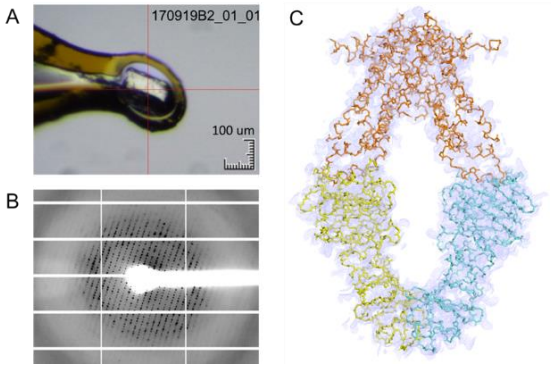

Figure 4. X-ray diffraction data and preliminary refined structure of TtFbpBC complex. **A**: Crystal of TtFbpBC. **B**: X-ray diffraction pattern of the TtFbpBC crystal. C: The fitting of electron density and the coordinates. The backbone of TMD is shown in sticks and colored in orange. The backbone of two identical NBDs are shown in sticks and colored in yellow and cyan, repectively.

In cryo-EM analysis, TtFbpBC was prepared using 0.01% lauryl maltose neopentyl glycol (LMNG) and 0.015% n-dodecyl-β-D-maltopyranoside (DDM). The cryo-EM analysis of TtFbpBC showed that both dimer and monomer could be observed in 0.01% LMNG but only monomer was observed in 0.015% DDM. The best resolution of TtFbpBC in cryo-EM analysis was 8.4 Å. TtFbpBC-A was prepared using 0.015% DDM. However, the cryo-EM analysis of TtFbpBC-A in 0.015% DDM showed that only the electron density of TtFbpBC was observed, which indicated that TtFbpBC-A was dissociated to TtFbpBC and TtFbpA under the cryogenic conditions used (**Table 1**).

Table 1. Samples observed by cryo-EM

Samples	Concentration	Additives	Detergent	Status	Resolution
FbpBC	15 mg/mL	1 mM ADP-Mg^{2+}	0.01% LMNG	Monomer and Dimer	8.4 Å
FbpBC	15 mg/mL	1 mM ADP-Mg^{2+}	0.015% DDM	Monomer	>> 8.4 Å
FbpBC-A	15 mg/mL	1 mM ADP-Mg^{2+}	0.015% DDM	Monomer (no TtFbpA)	8.4 Å

3) To inhibit the growth of Vibrio species, based on iron restriction via the inhibition of FbpA

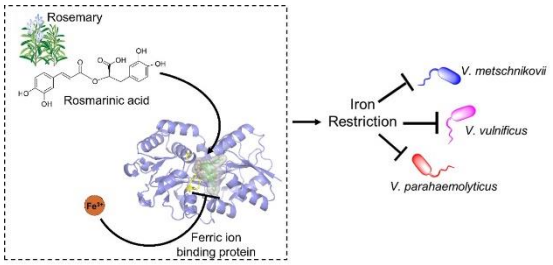

Figure 5. The mechanism of RA inhibiting the growth of *Vibrio* species.

To find bacteriostatic compounds that inhibit the function of VmFbpA and the growth of *V. metschnikovii*. The screening of VmFbpA-Fe^{3+} interaction inhibitors was performed using the water extracts of 20 different spices. In this study, rosemary and cinnamon extracts showed significant inhibitory activity. The UV-Vis spectral analysis showed that the inhibition of VmFbpA caused by rosemary extract is a specific reaction. Moreover, it was revealed that rosmarinic acid (RA) is the main compound responsible for the inhibition. The IC_{50} for RA to inhibit the interaction of 0.3 mM VmFbpA and Fe^{3+} is 800 ± 100 μM. ITC and docking simulation results demonstrated that RA binds to the VmFbpA at the Fe^{3+}-binding site and acts as a competitive inhibitor with a K_D of 8 μM. Since RA can reduce Fe^{3+} to Fe^{2+}, the fact that the Fe^{3+} release from VmFbpA by RA was caused by 1) the competitive inhibition of VmFbpA by RA and 2) the reduction of Fe^{3+} to Fe^{2+} by RA.

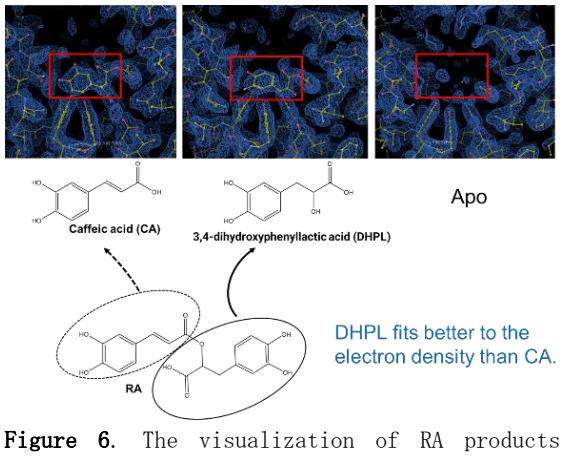

Figure 6. The visualization of RA products binding to VmFbpA.

In the antibacterial assay, the supplementation of a small compound significantly increased the inhibitory activity of RA on the growth of *V. metschnikovii*. However, RA with or without the small compound showed little effect on *E. coli*, which is more resistant to iron restriction. The results obtained in this study suggests that RA combined with the small compound would be a promising bacteriostatic agent against *V. metschnikovii* and other *Vibrio* spp. with fewer effects on indigenous gastrointestinal bacteria (**Figure 5**)[1].

Moreover, the crystal structure of apo VmFbpA and RA-VmFbpA was solved at the resolution of around 2.0Å. The product of RA bound to the iron-binding site of FbpA from *V. metschnikovii* was clarified, and the molecular mechanism by which rosmarinic acid inhibits iron uptake by Vibrio bacteria was successfully revealed (**Figure 6**). We are preparing a manuscript for the publication of this result.

＜引用文献＞

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5．主な発表論文等

〔雑誌論文〕　計1件（うち査読付論文　1件／うち国際共著　0件／うちオープンアクセス　1件）

1．著者名 Lu Peng、Sui Miaomiao、Zhang Mimin、Wang Mengyao、Kamiya Takehiro、Okamoto Ken、Itoh Hideaki、Okuda Suguru、Suzuki Michio、Asakura Tomiko、Fujiwara Toru、Nagata Koji	4．巻 22
2．論文標題 Rosmarinic Acid and Sodium Citrate Have a Synergistic Bacteriostatic Effect against Vibrio Species by Inhibiting Iron Uptake	5．発行年 2021年
3．雑誌名 International Journal of Molecular Sciences	6．最初と最後の頁 13010～13010
掲載論文のDOI（デジタルオブジェクト識別子） 10.3390/ijms222313010	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 －

〔学会発表〕　計2件（うち招待講演　0件／うち国際学会　1件）

1．発表者名 陸 鵬、王 夢瑶、神谷 岳洋、張 米敏、岡本 研、鈴木 道生、朝倉 富子、藤原 徹、永田 宏次
2．発表標題 海洋病原菌Vibrio metschnikoviili の鉄吸収と増殖を阻害する化合物のスパイス抽出物からの探索
3．学会等名 農芸化学会
4．発表年 2020年

1．発表者名 Peng Lu, Miaomiao Sui, Mimin Zhang, Mengyao Wang, Takehiro Kamiya, Ken Okamoto, Hideaki Itoh, Suguru Okuda, Michio Suzuki, Tomiko Asakura, Toru Fujiwara, Koji Nagata
2．発表標題 Identification of bacteriostatic agents by inhibiting the iron uptake protein, FbpA, from a marine-borne Gram-negative bacterium, Vibrio metschnikovii
3．学会等名 The 8th International Symposium on Metallomics (ISM-8)（国際学会）
4．発表年 2022年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

－

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