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研究課題名（和文）Towards a new type of antibiotic: elucidation of the activation signal and signal transduction mechanism of the type III secretion system of Shigella flexneri

研究課題名（英文）Towards a new type of antibiotic: elucidation of the activation signal and signal transduction mechanism of the type III secretion system of Shigella flexneri

研究代表者

ＣＨＥＵＮＧ　Ｍａｒｔｉｎ（Cheung, Martin）

沖縄科学技術大学院大学・量子波光学顕微鏡ユニット・技術員

研究者番号：９０８３２１６３

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研究成果の概要（和文）：III型分泌系先端複合体（TC）について、低温電子顕微鏡と質量分析計を駆使して研究した結果、次の2つの重要な知見が得られた：i)TCは3つの異なる亜単位から成り、その形態は不均質である。今回、初めてサブナノメートルの分解能（9A）で可視化し、TCが三量体であることを明らかにした。さらに、三量体内の各亜単位がそれぞれ異なる方向を向いていること、ii)感染時にT3SSが活性化すると、これまで知られていなかった補助的な複合体がTCに結合すること、などを明らかにした。我々はこの新たに同定された複合体がプレポア複合体であると考え、TCが標的細胞と相互作用する仕組みを説明できるのではないかと考えている。

研究成果の学術的意義や社会的意義
These results could be highly significant not only for our fundamental understanding of type III secretion system function but also for the development of novel antimicrobials. The newly complex may serve as an ideal drug target to prevent the infectivity of Shigella.

研究成果の概要（英文）：Utilizing cryo-EM and mass spectrometry to study the type III secretion system tip complex (TC), two key findings were made: i) the TC comprises three distinct subunits with heterogeneous morphologies. Prior to this study, the TC had never been visualized to better than 20 A resolution and was believed to be a homopentameric complex. By visualizing, for the first time, the TC to sub-nanometer resolution (app. 9 A) by cryo-EM, this work revealed the TC to be a trimeric complex. Furthermore, each subunit within the trimer assumes a distinct orientation; ii) a hitherto unknown auxiliary complex binds to the TC upon activation of the T3SS during infection. A pore contiguous with the TC is known to be inserted into target cell membranes during infection. We believe this newly identified complex to be a pre-pore complex and may explain how the TC is able to interact with target cells.

研究分野：Microbiology

キーワード：Shigella flexneri　Type III secretion　Tip complex　Cryo-EM

様　式　C－１９、F－１９－１、Z－１９（共通）

１．研究開始当初の背景

Shigella bacteria are the causative agents of shigellosis, a potentially fatal disease caused by infection and injury of the large intestine. The World Health Organization recognizes shigellosis as a global endemic, estimating some 165 million global cases per annum, and a significant health burden in developing countries. Alarmingly, numerous studies have documented the isolation of antibiotic resistant strains of *Shigella* spp. For example, Darton et al. (2018) recently reported the emergence of azithromycin-resistant *Shigella* strains in Southeast Asia. With the growing global health threat posed by antibiotic-resistant pathogens, there is an urgent need for new antimicrobials utilizing novel modes of action.

Type III secretion systems (T3SSs) are non-critical components of pathogenic bacterial cells; they are not required for basic cell function and survival but are utilized specifically during the infective cycle. Also, T3SSs are not present in commensal bacteria, such as those of the microbiota. An antimicrobial specifically targeting the T3SS could potentially attenuate a bacterium's capacity to infect without profoundly disturbing the microbiota, thus greatly reducing the potential for resistance development. Furthermore, two crucial components of the T3SS, a 50-nm needle and tip complex (TC), form an extracellular appendage that would be readily accessible to drugs. As such, the T3SS and subcomplexes thereof are highly promising targets for novel antimicrobials.

２．研究の目的

The T3SS, and thus infective cycle of *Shigella*, is only activated upon contact with target cells (i.e., gastrointestinal cells), before which it exists in a quiescent yet primed state. The aforementioned extracellular needle/TC appendage is thought to constitute the main component of this contact-mediated mechanism. Upon host cell contact, a purported activation signal is predicted to emanate from the TC to the rest of the system via the needle. In this way, the TC functions as a molecular 'on' switch for activation of the entire T3SS machinery. While this activation and signal transduction mechanism is predicated to occur through protein conformational changes in the needle/TC appendage, the precise mechanism has yet to be elucidated.

To investigate how T3SS activation occurs, we previously used high-resolution electron microscopy (EM) to solve the structures of the needle and TC to ~7 Å and ~20 Å resolutions, respectively (Fujii et al., 2012; Cheung et al., 2015). Although the structures determined in these studies intimated at how an activation signal may occur, the limited resolutions imposed by technical constraints hampered deeper insights. In the intervening years since our previous publications, advances in cryo-EM instrumentation and techniques have been significant. This project sought to take advantage of these advancements to tackle this subject once again.

３．研究の方法

This project utilized a hybrid approach of cryo-EM complemented with mass spectrometry (MS) to study the structures of T3SS complexes purified from *Shigella flexneri*. Of particular concern with this approach was the intactness and integrity of the purified complexes; for our data to meaningfully reflect the physiological structure and composition of the T3SS, it was imperative that protein degradation was kept to a minimum throughout the workflow of the study. Our experimental approach was as follows:

(1) Prior to purification, *Shigella flexneri* cultures were incubated with chemical crosslinker bissulfosuccinimidyl suberate (BS^3) in order to stabilize the structure of the needle/TC appendage. BS^3 is an extracellular crosslinker as it does not permeate into cells and thus permitted crosslinking of the needle/TC appendage without affecting our ability to affinity purify T3SS complexes (via a His-tag situated on an intracellular T3SS subunit). *Shigella flexneri* cultures were also incubated with dye Congo Red before purification in order to activate the complexes; Congo Red is a known artificial inducer of *Shigella flexneri*'s T3SS (i.e., activation without the need for target cell contact).

(2) T3SS complexes purified in the presence of BS^3 and Congo Red were compared with complexes purified without BS^3. The motivation behind this was to allow for the direct comparison of our data with prior studies. The vast majority of studies on the T3SS have been on complexes purified without particular emphasis on maintaining the integrity of

the needle/TC appendage. Indeed, we were only able to find one previous study which employed a crosslinking method to stabilize the needle/TC before purification.

(3) For cryo-EM analysis, purified near-native T3SS complexes were embedded in thin vitreous ice films within the holes of holey carbon transmission electron microscopy grids using our perpetually-hydrated graphene oxide approach (Cheung et al., 2018). High-resolution cryo-EM images of the ice-embedded T3SS complexes were acquired using a Falcon III direct detector-equipped Talos Arctica cryo-electron microscope operating at 200 kV. A bespoke computational approach using image processing packages SPIDER, cryoSPARC, and Relion was employed to produce three-dimensional maps of the needle/TC appendage.

(4) Activated T3SS complexes with or without BS^3 treatment were analyzed by high-resolution MS in order to determine the full complement of T3SS proteins. Furthermore, by analyzing the relative abundances of peptides from identified proteins, we were able to determine a rough picture of the stoichiometric relationships between T3SS components.

4．研究成果

The multifaceted approach employed in this study resulted in two key findings: i) the TC comprises three distinct subunits with heterogeneous morphologies (Fig. 1a). Prior to this study, the TC had never been visualized to better than 20 Å resolution and was believed to be a homopentameric complex. By visualizing, for the first time, the TC to sub-nanometer resolution (~9 Å) by cryo-EM, this work revealed the TC to be a trimeric complex. Furthermore, each subunit within the trimer assumes a distinct orientation, which implies either subunit heterogeneity or conformational dissimilation within a homotrimer; ii) a hitherto unknown auxiliary complex binds to the TC upon activation of the T3SS during infection (Fig. 1b). A pore contiguous with the TC is known to be inserted into target cell membranes during infection. We believe this newly identified complex to be a pre-pore complex and may explain how the TC is able to interact with target cells.

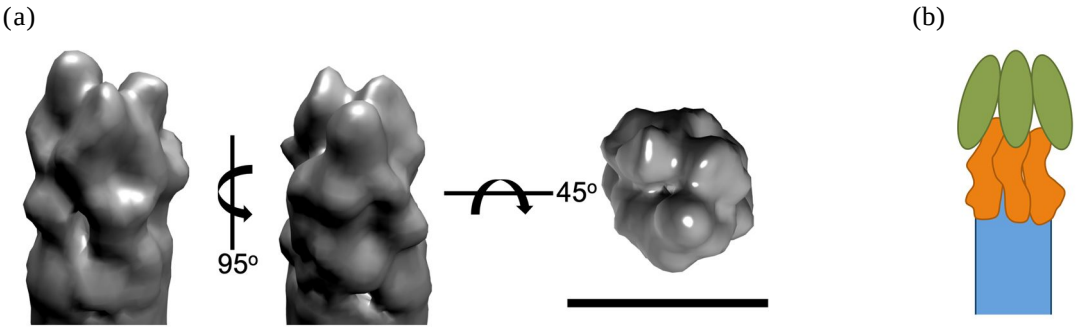

Fig. 1 (a) Cryo-EM map of the TC. A trimeric complex comprising morphologically distinct subunits can be seen. (b) Proposed pre-pore complex (green) bound to the TC (orange) following activation of the T3SS. Scale bar, 10 nm.

The precise mechanism by which the T3SS interacts with target cells has been a point of contention in the field. It has been shown that during infection, the TC binds to a pore embedded within the target cell membrane. However, the temporal formation and insertion of this pore has yet to be determined. Based on our findings, we contend that the newly identified auxiliary complex is a pre-pore complex that forms on top of the TC after T3SS activation but before contact with the target cell membrane. Upon contact with the target cell, the pre-pore is pushed into the membrane to form a transmembrane pore. This would explain when and how the pore is formed and how it interacts with the TC.

These results could be highly significant not only for our fundamental understanding of T3SS function but also for the development of novel antimicrobials. If the presence and role of this auxiliary complex can be confirmed, it may serve as an ideal drug target to prevent the activation of the T3SS and hence infectivity of *Shigella*. These findings warrant further investigation.

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