様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

科学研究費助成事業　　研究成果報告書

令和　５ 年　 ５ 月 ２３ 日現在

機関番号：１４４０１
研究種目：若手研究
研究期間：2020～2022
課題番号：２０Ｋ１６２４２

研究課題名（和文）Characterization of unstudied RND-type multidrug efflux pumps from pathogenic microorganisms

研究課題名（英文）Characterization of unstudied RND-type multidrug efflux pumps from pathogenic microorganisms

研究代表者

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交付決定額（研究期間全体）：（直接経費）　　3,200,000円

研究成果の概要（和文）：複数のグラム陰性菌からRND型多剤排出ポンプをクローニングし、発現させ、数種のRND型多剤排出ポンプの相違点と類似点を丹念に調べた上で変異を導入してきた。その結果、RND型多剤排出ポンプの機能、その進化、保存、機能に関して、多剤耐性分野に関連し、重要な興味深い新知見を得ることができた。この研究期間中に、複数の査読付きジャーナルに研究論文（およびレビュー記事）を発表した。

研究成果の学術的意義や社会的意義
We expanded our understanding of multidrug resistance and provided novel insights into the function and adaptation of RND pumps. This has social significance as it contributes to healthcare strategies in combating multidrug resistance and addresses the global health challenge of drug resistance.

研究成果の概要（英文）：Multidrug efflux pumps belonging to the RND superfamily from multiple Gram-negative bacteria have been cloned and expressed, and mutations have been introduced after meticulously studying the differences and similarities of hundreds of pumps. The results show interesting novel findings relevant and important to the field of multidrug resistance regarding the function of RND-type multidrug efflux pumps, their evolution, their conservation, and their function. During this research period, we have published research papers (as well as review articles) in multiple peer-reviewed journals.

研究分野：細菌学関連

キーワード：Multidrug resistance　Pathogens　Bacteria　Transporters　Phylogenetics　Evolution　Adaptation　Antibiotics

様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９（共通）

１．研究開始当初の背景

Multidrug resistance (MDR) is a major problem in global health today. One of the main factors of MDR in Gram-negative pathogens (such as *Escherichia coli*, *Salmonella*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*) is the over-expression of drug efflux pump proteins, especially those belonging to the resistance-nodulation-division (RND) superfamily, such as AcrB (Zwama, M. *et al.*, 2018, Nature Comm.). AcrB from *E. coli* has been functioning as a probe to understand drug export by RND-type transporters (Zwama, M. & Yamaguchi, A., 2018, Res. Microbiol.). We have recently found that an ancient pump from *Haemophilus influenzae* exports a similar range of substrates as more newly evolved AcrB from *E. coli*, apart from some physiologically relevant compounds (Zwama, M. *et al.*, 2019, Comm. Biol.). However, there is not much known about the many RND-type efflux pumps from multiple bacteria, which need to be studied to understand multidrug recognition and tripartite-complex formation. We set out to investigate the adaptation of RND-type pumps and the mechanisms of tripartite formation (RND, PAP, and OMP). We need this information to develop novel antibiotics and (universal) efflux pump inhibitors.

２．研究の目的

Multidrug resistance (MDR) is one of the major health issues worldwide, and resistant pathogens are increasing in number, especially during the COVID-19 pandemic, due to misusage and over usage of antibiotics and other reasons. Over-expression of RND-type multidrug efflux pumps has been attributed to MDR, as these pumps can recognize and expel a wide range of structurally diverse compounds. With this research, we sought to investigate multiple topics to guide the development of novel antibiotics and inhibitors.

Firstly, we investigated 15 RND-type efflux pump complexes, expressed together with their tripartite colleagues: the Periplasmic Adaptor Proteins (PAPs) and the Outer Membrane Channel tunnel proteins (OMPs). With this research, we aimed to understand universal binding mechanisms between the RND, PAP and OMP domains.

Secondly, we investigated the adaptation of RND efflux pumps under antibiotic pressure. We compared hundreds of pumps and analyzed conserved and variable regions. In addition, we investigated clinical mutations, which increased the efflux pump's ability to export macrolide antibiotics. This research aimed to understand adaptation under physiological and hospital environments to guide antibiotics usage in a clinical setting.

３．研究の方法

We cloned multiple efflux pumps from different organisms into expression vectors. In addition, the three genes of the three tripartite members (RND, PAP, and OMP) were designed and codon optimized for *E. coli*. Drug efflux activity was analyzed by Minimum Inhibitory Concentration (MIC) experiments on LB agar plates and liquid LB supplemented with antibiotics. We altered RND/PAP/OMP genes and combinations by using PCR. We have performed these experiments and obtained interesting results; however, they are currently still ongoing, and future reports and publications will report the final findings.

As for the other part of the research, we introduced mutations similar or identical to amino acid substitutions found in clinical strains with higher drug resistance and analyzed the effects by MIC and Kirby-Bauer disk diffusion susceptibility tests. We also performed an analytical study on these mutations and the general adaptive behavior of RND-type MDR pumps. We computationally compared hundreds of pumps to determine variable and conservative domains in RND-type pumps by combining multiple sequence alignment (Clustal Omega), followed by manual and computational (ConSurf) analysis, combined with literature research.

４．研究成果

One of the major studies in this research project was the analysis of multiple efflux pumps. To be brief, this research has been successful and yielded exciting results; however, as this is yet unpublished and ongoing, we currently refrain from publishing the results thus far in this report. However, the experiments have been fruitful, and we are looking forward to continuing this research and publishing it promptly.

Furthermore, we were interested in the adaptability of RND-type efflux pumps. In the literature, we found that azithromycin resistance was increased in *Salmonella enterica Serovars* Typhi and Paratyphi A clinical strains. These strains had either the R717Q or R717L mutation in the efflux pump AcrB. We also found clinical mutation R714G substitution in MtrD from clinically isolated *Neisseria gonorrhoeae* in literature. We investigated R717Q/L in *E. coli* AcrB (AcrB-Ec), closely related to Salmonella AcrB, expressed in *E. coli*. We compared the effects to other clinical mutations, namely, K823E/N (causing enhanced AZM resistance by MtrD) and G288D (causing fluoroquinolone resistance in a *S. Typhimurium* clinical strain).

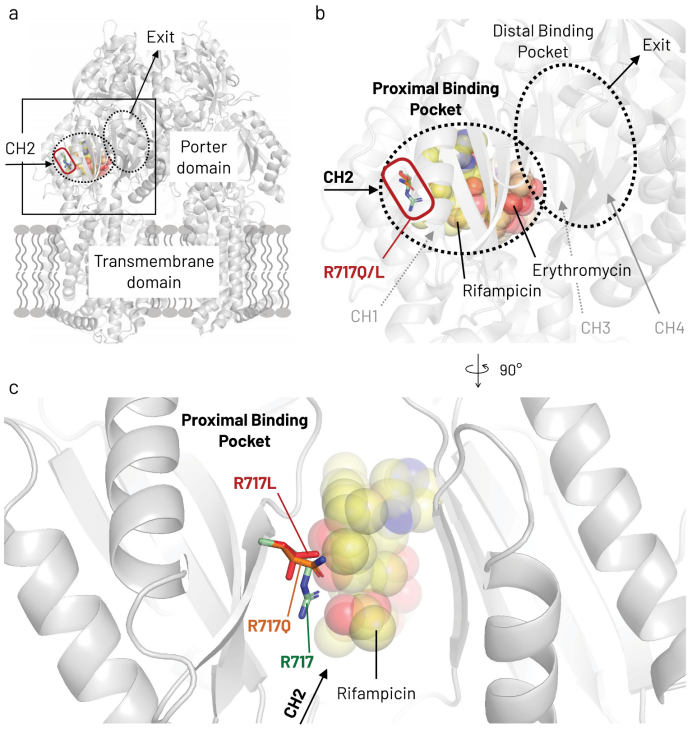

Figure 1. Location of Arg717 and the R717Q/L amino acid substitutions in AcrB-Ec.

We found that the mutations substantially increased the MICs for macrolides erythromycin, azithromycin, and azithromycin. The highest increase was seen for R717L expelling azithromycin. Interestingly, cloxacillin and novobiocin MICs were decreased by 2-fold for both mutations.

	Minimum inhibitory concn (MIC) (μg mL^{-1})[b]																			
	Planar aromatic cations					Macrolides			β-lactams		Quinolones						Other		Bile salts	
Strain[c]	EtBr	MtB	ACR	CV	BZK	ERY	AZM	CLR	CLX	ATM	NAL	NOR	CIP	OFX	LVX	MXF	NOV	MIN	Mix	DEOX
Vector	16	16	16	2	4	2	<0.5	2	1	1/16	1	1/128	1/256	1/64	1/128	1/256	4	0.125	2,500	625
WT	512	>1,024	256	8–16	128	128	16	128	256	1/32	4	1/32	1/64	1/16	1/32	1/16	256	1	>20,000	10,000
R717Q	512	>1,024	256	8	128	**256**	**64**	**>256**	*128*	**0.125**	4	1/32	1/64	**0.125**	**1/16**	**0.125**	*128*	1	>20,000	10,000
R717L	**1,024**	>1,024	256	8	128	**256**	**128**	**>256**	*128*	1/32	4	1/32	1/64	**0.125**	1/32	**0.125**	*128*	1	>20,000	10,000
G288D	*256*	*1,024*	*128*	8	*64*	*64*	*4*	*64*	*128*	**0.125**	4	1/32	1/64	1/16	1/32	1/16	*64*	1	>20,000	**20,000**
E826K	512	>1,024	*128*	8	128	128	*4*	*64*–128	256	**1/16**	4	1/32	1/64	1/16	1/32	1/16	256	1	>20,000	10,000
E826N	512	>1,024	256	8	128	128	16	128	256	**1/16**	4	1/32	1/64	1/16	1/32	1/16	256	1	>20,000	**20,000**

Table 1. Antimicrobial susceptibility of AcrB-Ec expressing cells with several substitutions.

Similar results are seen in the liquid MICs and the Kirby-Bauer disk diffusion susceptibility tests. Besides increased macrolide and fluoroquinolone resistance, we observed decreased MICs for CLX and NOV. Therefore, combining multiple antibiotics to treat typhoid and paratyphoid fever may be clinically interesting to mitigate resistance and enhance treatment.

For example, a combination of beta-lactams and azithromycin may enhance the treatment of *Salmonella* infections. These results have been published in AAC (1).

Furthermore, we have analyzed multiple pumps to investigate variable and conservative regions. The results show that the transmembrane domain of RND-type efflux pumps are highly conserved, while the porter (periplasmic) domain is relatively variable.

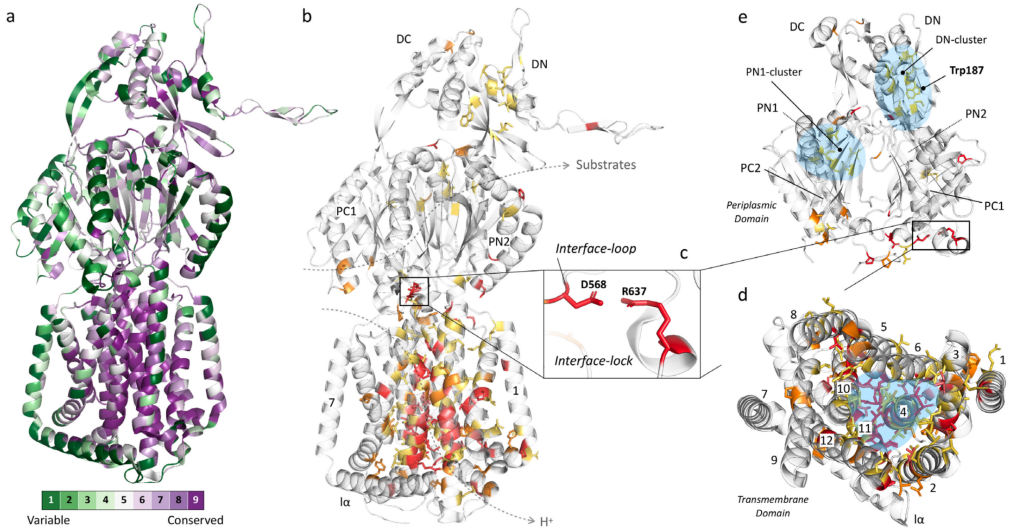

Figure 2. Heat maps of conservation based on 135 sequences of RND efflux pumps.

In this research, we investigated not only variability and conservative domain differences but also compared binding pockets to investigate differences in substrate specificity. We thoroughly identified residues part of the multiple binding pockets and regions with the RND-type efflux pumps and attributed specific amino acids to some drug recognition specificity, however, it generally was not always clear that one or more amino acid substitutions attributed to some specificities, such as aminoglycoside or monobactam selectivity, which we investigated further and attributed to sums of charged and hydrophobic residues in the binding pockets (electrostatic and hydrophobic environments). We also found that narrow-spectrum beta-lactams were the only class of antibiotics that were exported by all investigated pumps from several bacteria. We attributed this find to the possibility of the completely conserved residue Y327 (numbered as in AcrB-Ec). Lastly we created an overview of recent amino acid substitutions in the RND-type pumps found in clinical isolates, including the one from our other research: R717Q/L (1). The results are published in Antibiotics (2).

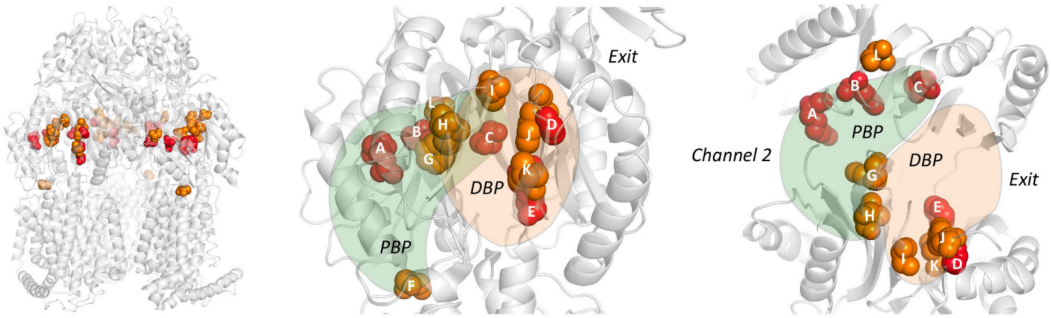

Figure 3. Upcoming gain-of-function mutations in RND-type efflux pumps.

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2) Martijn Zwama & Kunihiko Nishino. Ever-Adapting RND Efflux Pumps in Gram-Negative Multidrug-Resistant Pathogens: A Race against Time. *Antibiotics* **10**, 774 (2021).

５．主な発表論文等

〔雑誌論文〕　計8件（うち査読付論文　7件／うち国際共著　6件／うちオープンアクセス　6件）

１．著者名 Zwama Martijn、Nishino Kunihiko	４．巻 10
２．論文標題 Ever-Adapting RND Efflux Pumps in Gram-Negative Multidrug-Resistant Pathogens: A Race against Time	５．発行年 2021年
３．雑誌名 Antibiotics	６．最初と最後の頁 774～774
掲載論文のDOI（デジタルオブジェクト識別子） 10.3390/antibiotics10070774	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 該当する

１．著者名 Zwama Martijn、Nishino Kunihiko	４．巻 12.16
２．論文標題 Proximal binding pocket Arg717 substitutions in Escherichia coli AcrB cause clinically relevant divergencies in resistance profiles	５．発行年 2021年
３．雑誌名 bioRxiv	６．最初と最後の頁 473095
掲載論文のDOI（デジタルオブジェクト識別子） 10.1101/2021.12.16.473095	査読の有無 無
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 該当する

１．著者名 Zwama Martijn、Nishino Kunihiko	４．巻 1
２．論文標題 Proximal Binding Pocket Arg717 Substitutions in Escherichia coli AcrB Cause Clinically Relevant Divergencies in Resistance Profiles	５．発行年 2022年
３．雑誌名 Antimicrobial Agents and Chemotherapy	６．最初と最後の頁 e02392-21
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３．雑誌名 Frontiers in Microbiology	６．最初と最後の頁 737288
掲載論文のDOI（デジタルオブジェクト識別子） 10.3389/fmicb.2021.737288	査読の有無 有
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1．著者名 Koga Naoki、Hosomi Takuro、Zwama Martijn、Jirayupat Chaiyanut、Yanagida Takeshi、Nishino Kunihiko、Yamasaki Seiji	4．巻 11
2．論文標題 Identification of Genetic Variants via Bacterial Respiration Gas Analysis	5．発行年 2020年
3．雑誌名 Frontiers in Microbiology	6．最初と最後の頁 581571
掲載論文のDOI（デジタルオブジェクト識別子） 10.3389/fmicb.2020.581571	査読の有無 有
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2．論文標題 タンパク質構造情報を利用した細菌多剤排出トランスポーターの機能解析	5．発行年 2020年
3．雑誌名 Bio Clinica	6．最初と最後の頁 63～68
掲載論文のDOI（デジタルオブジェクト識別子） なし	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 －

1．著者名 Yamasaki Seiji、Koga Naoki、Zwama Martijn、Sakurai Keisuke、Nakashima Ryosuke、Yamaguchi Akihito、Nishino Kunihiko	4．巻 66
2．論文標題 Spatial Characteristics of the Efflux Pump MexB Determine Inhibitor Binding	5．発行年 2022年
3．雑誌名 Antimicrobial Agents and Chemotherapy	6．最初と最後の頁 e00672-22
掲載論文のDOI（デジタルオブジェクト識別子） 10.1128/aac.00672-22	査読の有無 有
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1．著者名 Seiji Yamasaki, Martijn Zwama, Koga Naoki, Keisuke Sakurai, Ryosuke Nakashima, Akihito Yamaguchi, Kunihiko Nishino	4．巻 5
2．論文標題 Inhibitor binding pits analysis of bacterial multidrug efflux transporters for new drug development	5．発行年 2022年
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オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 －

〔学会発表〕　計2件（うち招待講演　2件／うち国際学会　1件）

1．発表者名 Martijn Zwama, Akihito Yamaguchi, Kunihiko Nishino
2．発表標題 Phylogenetic analysis and environmental adaption of multidrug efflux pumps
3．学会等名 The 93rd annual meeting of Japanese Society for Bacteriology（招待講演）（国際学会）
4．発表年 2020年

1．発表者名 Martijn Zwama, Ryosuke Nakashima, Seiji Yamasaki, Kunihiko Nishino
2．発表標題 The increasingly pressing role of RND-type efflux pumps in multidrug resistant pathogens
3．学会等名 10th imec Handai International Symposium（招待講演）
4．発表年 2021年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

https://www.sanken.osaka-u.ac.jp/labs/mid/Site/Welcome.html

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