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研究課題名（和文）Mechanism, evolution and regulation of multidrug resistance

研究課題名（英文）Mechanism, evolution and regulation of multidrug resistance

研究代表者
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交付決定額（研究期間全体）：（直接経費）　　2,300,000円

研究成果の概要（和文）：本研究では、RND型多剤排出ポンプの進化的解析を行い、薬剤および排出ポンプ阻害剤の特異性に関する知見を提供できました。
古い排出ポンプである（AcrB-Hi）は進化したポンプ（AcrB-Ec）と同様に幅広い薬剤の排出を、インフルエンザ菌と大腸菌により発見しました。AcrB-HiはAcrB-Ecとは対照的に、胆汁酸をほとんど排出できず、AcrB-Ecが阻害剤により機能を抑制されるのに対し、AcrB- Hiは抑制されませんでした。インフルエンザ菌の特定の薬物に対する抗菌薬感受性は、外膜タンパク質の存在により説明できます。
これらの結果は、新規抗菌薬と排出ポンプ阻害剤の開発に重要な意味をもたらします。

研究成果の学術的意義や社会的意義
The results from this research provide important insights for the development of novel antibiotics and efflux pump inhibitors. This research is of importance not only for our scientific understanding of MDR, but also for our search for new antibiotics to benefit global human health.

研究成果の概要（英文）：With this research, I was able to analyze the evolution of RND-type multidrug efflux pumps and provide insights into drug and efflux pump inhibitor specificity. I found that an ancient efflux pump (AcrB-Hi) can export the same range of antibiotics and an evolved pump (AcrB-Ec), from different bacteria, Haemophilus influenzae and Escherichia coli, respectively. However, there were some important differences observed. AcrB-Hi was not able to export bile salts efficiently, as opposed to AcrB-Ec. Additionally, AcrB-Hi was uninhibited by an efflux pump inhibitor, which inhibited AcrB-Ec completely. The drug sensitivity to certain drugs of H. influenzae cells could be explained by the presence of a large outer membrane protein. These results provide important insights for the development of novel antibiotics and efflux pump inhibitors.

研究分野：細菌学

キーワード：AcrB　Evolution　Multidrug resistance　Efflux pump　Pathogens　Phylogenesis　Haemophilus influenzae　Efflux pump inhibitor

１．研究開始当初の背景

Today, multidrug resistance (MDR) is a major problem in global health. One of the main factors of MDR in Gram-negative pathogens (such as *Escherichia coli* and *Haemophilus influenza*) is the over-expression of drug efflux pump proteins. The protein pumps belong 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.), however little is known about many other RND-type efflux pumps from many other microorganisms.

２．研究の目的

To understand the drug recognition by RND-type transporters, it would help to study different drug efflux pumps from different microorganisms. Different bacteria have different sets of amino-acids in their drug binding pockets, and it is therefore not yet completely clear how antibiotics are selected and exported by these type of efflux pumps.

An example of this is the hydrophobic trap, which does not only bind several antibiotics, but is also the key area of this protein (AcrB from *E. c*
oli, and MexB from *Pseudomonas aeruginosa*) for the development of efflux pump inhibitors (EPIs). This pocket (in the aforementioned proteins) consist of several phenylalanine (Phe) residues, and is therefore also named the Phe-rich pit. EPIs bind strongly to the Phe residues, inhibiting the active efflux of antibiotics, and therefore resensitizing pathogens to antibiotics.

However, not all efflux pumps have the same pit, and I wanted to investigate the pits, the effectiveness of EPIs, and the drug recognition spectrum, in the context of ancient and evolved RND-type transporters (phylogenesis).

３．研究の方法

For this research I set out to investigate the phylogenetic relationships between many RND-type efflux transporters from e.g. *E. coli*, *H. influenzae*, *Acinetobacter baumannii* and *Legionella pneumophila*. By multiple sequence alignment I phylogenetically organized around 300 RND-type genes from Gram-negative proteobacteria. From this data I could observe phylogenetic relationships and obtain insights in the evolution of efflux pumps; which were in early branched clusters and which were at the very end. Based on this data, I selected two RND-type transporters, an ancient and an evolved (adapted) pump. The genes of the pumps were amplified by PCR and cloned into plasmids. These plasmids were transformed in *E. coli* cells in order to directly compare the pumps in the same environment. From these two pumps, I analyzed the residues in the drug binding pockets and the hydrophobic trap, and determined the differences and similarities of their drug efflux spectrum by Minimum Inhibition Concentration (MIC) experiments. Amino acids were mutated by modifying the gene by PCR and tested for altered EPI effectiveness and drug export. Additionally, an outer membrane channel was amplified, cloned and expressed to see its effect on drug sensitivity.

4．研究成果

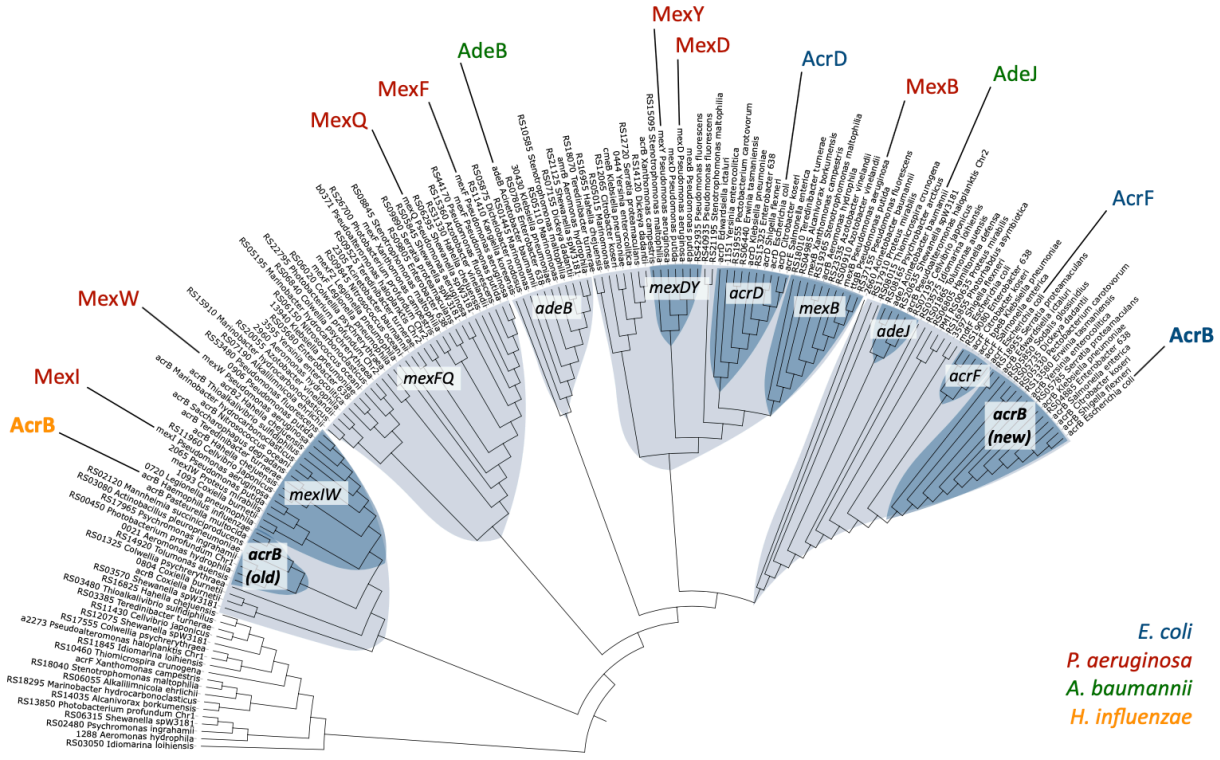

Fig. 1. The phylogenetic relationship between 300 RND efflux pumps. AcrB-Hi is located on the left (yellow) and AcrB-Hi is located on the right (blue).

By phylogenetically analyzing around 300 genes, I found that *H. influenzae* AcrB (AcrB-Hi) was a relatively ancient efflux pump, and that *E. coli* AcrB (AcrB-Ec) was one of the mostly adapted pump, from the analyzed RND-type homologous efflux pumps (Fig. 1). AcrB-Hi could expel the same antibiotics as evolved AcrB-Ec, including certain antibiotics from the β-lactams class of antibiotics (some of which are used as first-line drugs to treat *H. influenzae* infections).

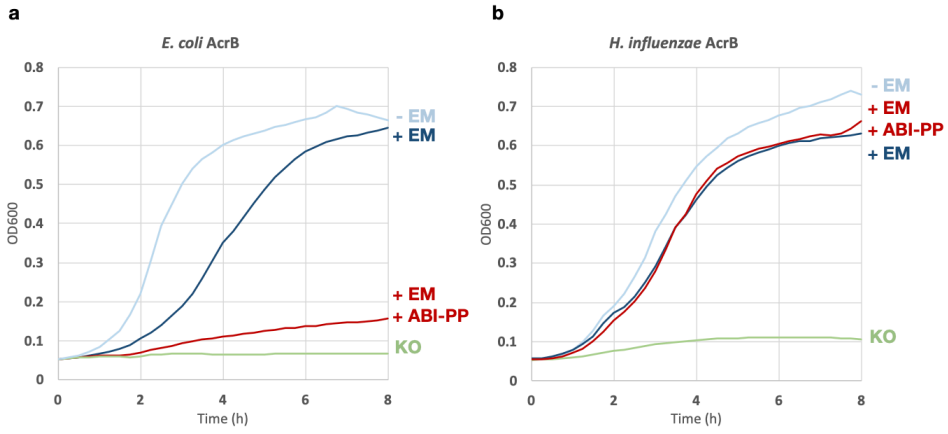

Fig. 2. The effect of EPI ABI-PP on the cell growth of E. coli in presence and absence of antibiotics, while expressing either AcrB-Ec (a, left) or AcrB-Hi (b, right). EM + EPI is shown in red. Em; erythromycin.

However, I found some interesting differences, too. AcrB-Hi could not efficiently expel bile salts, which are abundant in the natural environment of *E. coli* (which could very effectively export bile salts), but not in that of *H. influenzae*. Additionally, AcrB-Hi was completely uninhibited by the efflux pump inhibitor ABI-PP (Fig. 2). This EPI binds strongly to the hydrophobic Phe-rich trap of AcrB-Ec, by which it inhibits the active efflux of antibiotics. I analyze the pits of both pumps, and found that AcrB-Hi does not have a Phe-rich pit, which could explain why ABI-PP was unable to inhibit the efflux pump (Fig. 3).

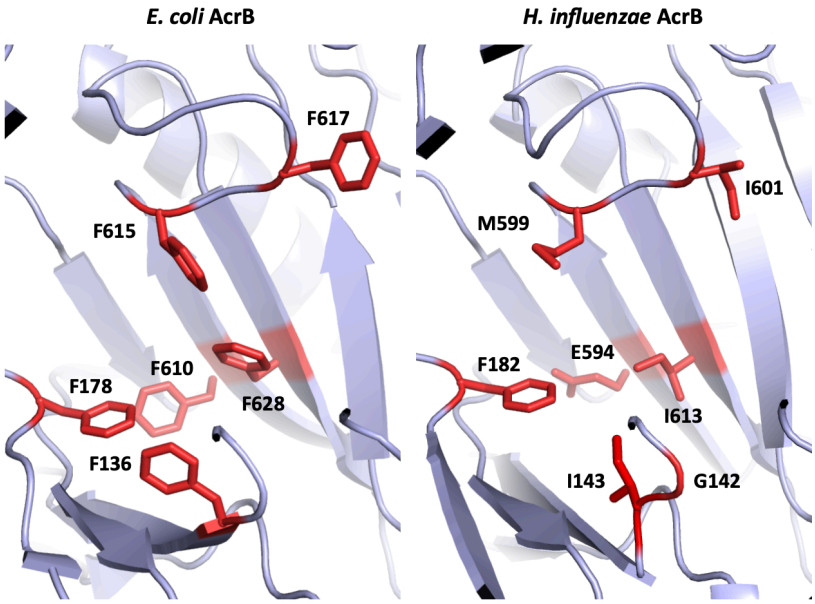

Fig. 3. The hydrophobic trap of AcrB-Ec (left) and AcrB-Hi (right). The EPI binding pit of AcrB-Ec contains many Phe-residues, which are absent in the pit of AcrB-Hi.

Lastly, AcrB-Hi could expel β-lactams very effectively, while these drugs are used to treat *H. influenzae* infections. Shortly, we observed a high MIC for AcrB-Hi expressing *E. coli* cells, but the MICs for *H. influenzae* cells were the same as AcrB-KO cells. I investigated this phenomenon by expressing the wide *H. influenzae* outer membrane channel OmpP2 in *E. coli* cells, and observed that the MIC values were significantly lowered for β-lactams and novobiocin, directly explaining why *H. influenzae* is sensitive to these drugs.

５．主な発表論文等

〔雑誌論文〕　計3件（うち査読付論文　3件／うち国際共著　2件／うちオープンアクセス　1件）

１．著者名 Zwama Martijn、Yamaguchi Akihito	４．巻 169 (7-8)
２．論文標題 Molecular mechanisms of AcrB-mediated multidrug export	５．発行年 2018年
３．雑誌名 Research in Microbiology	６．最初と最後の頁 372～383
掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/j.resmic.2018.05.005	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 該当する

１．著者名 Zwama Martijn、Yamaguchi Akihito、Nishino Kunihiko	４．巻 2 (1)
２．論文標題 Phylogenetic and functional characterisation of the Haemophilus influenzae multidrug efflux pump AcrB	５．発行年 2019年
３．雑誌名 Communications Biology	６．最初と最後の頁 1～11
掲載論文のDOI（デジタルオブジェクト識別子） 10.1038/s42003-019-0564-6	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 Seiji Yamasaki, Martijn Zwama, Ryosuke Nakashima, Akihito Yamaguchi, Kunihiko Nishino	４．巻 2
２．論文標題 Mutational analysis of bacterial efflux transporters contributing to antibiotic resistance	５．発行年 2019年
３．雑誌名 Precision Medicine	６．最初と最後の頁 60～65
掲載論文のDOI（デジタルオブジェクト識別子） -	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 －

〔学会発表〕　計3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 The fight against antibiotic resistance bacteria: understanding multidrug efflux pumps
３．学会等名 iMEC Handai International Symposium, Leuven, Belgium（招待講演）
４．発表年 2018年

１．発表者名
Martijn Zwama, Akihito Yamaguchi, Kunihiko Nishino

２．発表標題
The defence mechanism of Haemophilus influenzae—a synergy between ancestral efflux pump AcrB and porin OmpP2

３．学会等名
Hong Kong University, Hong Kong

４．発表年
2019年

１．発表者名
Martijn Zwama, Akihito Yamaguchi, Kunihiko Nishino

２．発表標題
Phylogenetic analysis and environmental adaptation of multidrug efflux pumps

３．学会等名
The 93rd annual meeting of Japanese Society for Bacteriology, Nagoya, Japan（招待講演）（国際学会）

４．発表年
2020年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

ホームページ等
https://www.sanken.osaka-u.ac.jp/labs/mid/Site/Welcome.html

６．研究組織

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