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## Preface

Multidrug exporters are intrinsic membrane proteins widely distributed in bacteria. They play as cellular self-defense mechanisms and have some other physiological roles. They contribute to bacterial tolerance against antibiotics. When they are over-expressed, they cause multidrug resistance. Multidrug-resistant pathogens have caused great difficulties in modern chemotherapy. There have been no clinically useful drugs against bacterial multidrug exporters. They can be classified into three major families: ABC, MFS, and RND. ABC-type exporters are ATP-hydrolysis-coupled transporters, which are major multidrug exporters in mammalian models, but in microorganisms, ABC-type exporters play a minor role in drug resistance. MFS-type exporters are drug/proton antiporters, which are major drug exporters in multidrug resistant Gram-positive pathogens. MATE-type is a subfamily of MFS-type transporters. RND-type exporters are multidrug exporters characteristic in Gram-negative bacteria, which consist of a tripartite complex passing through cytoplasmic membrane, periplasm, and the outer membrane. The most characteristic common properties of multidrug exporters are their extremely broad substrate specificity. For example, major multidrug exporter AcrAB-TolC in *E. coli* exports both aromatic and aliphatic compounds including cationic, anionic, twitter ionic, and neutral compounds. However, it has own specificity. It exports oxacillin but does not export carbenicillin. Clarifying the mechanism of multidrug exporters, specifically as they export such a wide range of drugs and toxic compounds, is one of major challenges posed to our modern science.

Elucidation of multidrug efflux mechanisms has been greatly advanced mainly by structure determination of bacterial multidrug exporters during the last decade. Prior to the 2000s, no transporters' molecular structures were solved. Now, the crystal structures of more than ten multidrug exporters including all three types (RND, MFS, and ABC) have been solved. This success is supported by the advancement of the technology to express a large amount of tagged membrane proteins, development of detergents to solubilize membrane proteins, large-scale purification using affinity chromatography, as well as development of the method for crystallization including facilitator of crystallization such as DARPIn and monobody. As a result, we have been able to understand the outline of the structural basis of multidrug recognition and multidrug efflux mechanisms.

In the first part of this book, we present protocols to introduce marvelous success in determining multidrug exporter structures during the last decade. Chapters 1 and 2 show the protocols for determination of the high-resolution structures and the ligand-binding structures of RND-type multidrug exporter AcrB, which are the pioneering works for the structural study of bacterial multidrug exporters. They revealed that the multidrug efflux is mediated by the functional rotation mechanism of an asymmetric homo-trimer, and the structural basis of the multidrug recognition is a multisite drug binding. RND-type transporters are tripartite complexes composed of cell membrane transporters, outer membrane channels, and adaptor proteins. Chapter 3 describes the crystal structure of another interesting type multidrug exporter, MATE-type, bound with its inhibitors. It also describes the novel artificial and systematic transporter-inhibitor construction method. Chapter 4 shows the first crystal structure of the complex of a cell membrane transporter and an adaptor protein two-part complex. Unlikely to the conventional sense, an adaptor protein is not just an adaptor but the hexamer of the adaptor proteins makes a periplasmic channel that

connects between a cell membrane transporter and an outer membrane channel. Chapter 5 shows the first whole structure of a tripartite complex of the RND-type exporter by cryo-EM images. Chapter 6 shows another strong tool for structure determination of a membrane transporter using NMR spectroscopy. Chapter 7 describes one of the powerful tools for crystallization of a membrane transporter.

In order to understand how to work the molecular structures of multidrug exporters to transport drugs, biochemical and bioengineering analysis is absolutely necessary. In the second section, we will show a few important examples of the numerous biochemical and genetic studies of exporters. With respect to the RND-type transporters, reconstitution studies for whole tripartite complex are tremendously difficult because the complex penetrates through two membranes. Chapter 8 shows the breakthrough achievement of the reconstitution of the tripartite complex and the measurement of multidrug transport by RND-type transporters through artificial membranes. Chapter 9 proves the functional rotation mechanism of drug export mediated by RND-type exporters using artificial covalently linked trimers of cell membrane transporter. Chapter 10 shows the intra-protein drug translocation pathway by introducing site-directed mutagenesis.

In Part III, we show the computational analysis of how to work the exporter structure and how to predict a novel efflux pump. Chapter 11 shows an excellent example of the molecular dynamic simulations of the RND-type exporter. Chapter 12 shows a transcriptomic approach to identify novel efflux pumps.

Multidrug exporters play a variety of physiological roles under various expression controls. In Part IV, we show how to regulate the exporter expression and biomedical roles of exporters. Chapters 13 and 14 show the condition and the mechanism of expression regulation of the bacterial multidrug exporters. Chapter 15 shows the identification of the expression regulators. Chapter 16 shows high-throughput screening of multidrug efflux system useful for identification of the physiological role of exporters.

Finally, in the Part V, we show the advanced technologies useful for future works of multidrug exporters. Chapter 17 shows single-molecule analysis of membrane transporter activity using artificial membranes stretched on a microchamber. Chapter 18 shows a single-cell efflux assay method using femtoliter droplet arrays. Chapter 19 shows reconstitution and active transport assay using liposomes including V-ATPase for a proton-motive force supplier.

The field of multidrug exporter studies is a fast developing field of science. I hope this collection of protocols will contribute to the work of many researchers studying multidrug exporters. I would like to thank all authors for sharing their valuable experience and insights with the research community at large. I would like to thank the series editor Dr. John Walker for help with reviewing the book.

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