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Biochemical Characterization of the Flavivirus-resistance Mouse ABCF3 Protein and a Review of the Antibiotic Resistance and Dissemination Mechanisms in Bacteria

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BIOCHEMICAL CHARACTERIZATION OF THE FLAVIVIRUS-RESISTANCE MOUSE ABCF3 PROTEIN AND A REVIEW OF THE ANTIBIOTIC RESISTANCE AND DISSEMINATION MECHANISMS IN BACTERIA

by

ELIZABETH PETERSON

Under the Direction of Parjit Kaur, PhD

ABSTRACT

Mammalian ATP-binding cassette subfamily F member 3 (ABCF3) is a class 2 ABC protein that has previously been identified as a partner of the mouse flavivirus resistance protein 2′,5′-oligoadenylate synthetase 1B (OAS1B). The functions and natural substrates of ABCF3 are currently not known. In Chapter 1 of this study, we show that purified ABCF3 is an active ATPase. Binding analyses with a fluorescent ATP analog TNP-ATP suggested unequal contributions by the two nucleotide-binding domains. We further showed that ABCF3 activity is increased by lipids, including sphingosine, sphingomyelin, platelet-activating factor, and lysophosphatidylcholine. However, cholesterol inhibited ABCF3 activity, whereas alkyl ether

lipids either inhibited or resulted in a biphasic response, suggesting small changes in lipid structure differentially affect ABCF3 activity. Point mutations in the two nucleotide-binding domains of ABCF3 affected basal and sphingosine-stimulated ATPase activity differently, further supporting different roles for the two catalytic pockets. We propose a model in which pocket 1 is the site of basal catalysis, whereas pocket 2 engages in ligand-stimulated ATP hydrolysis. Co-localization of the ABCF3–OAS1B complex to the virus-remodeled endoplasmic reticulum membrane has been shown before. We show that co-expression of ABCF3 and OAS1B in bacteria alleviated growth inhibition caused by expression of OAS1B alone, and significantly enhanced OAS1B levels, indirectly showing interaction between these two proteins in bacterial cells. As viral RNA synthesis requires large amounts of ATP, we conclude that lipid-stimulated ATP hydrolysis may contribute to the reduction in viral RNA production characteristic of the flavivirus resistance phenotype.

Chapter 2 of this dissertation provides a comprehensive review of the major known antibiotic resistance mechanisms, including the function of ABC proteins, found in producer soil bacteria and discusses different horizontal gene transfer mechanisms that may play a role in the dissemination of resistance genes from producer and non-producer environmental bacteria to pathogenic bacteria in clinical settings. Many bacterial and eukaryotic ABC proteins are polyspecific in nature and are capable of transporting structurally diverse compounds, including drugs and lipids. These proteins are responsible for intrinsic or acquired multidrug resistance, which can also spread to pathogenic organisms through the horizontal transfer mechanisms discussed in this review.

INDEX WORDS: ABCF3, OAS1B, West Nile virus (WNV), flavivirus-resistance, glycerophospholipids, alkyl ether lipids, sphingolipids, fluorescence, TNP-ATP binding, ATP hydrolysis, self-resistance mechanisms, antibiotic resistance, *Streptomyces*, horizontal gene transfer, resistance gene dissemination

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DEDICATION

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LIST OF ABBREVIATIONS

ABC, ATP-binding cassette superfamily
ABCF3, ATP-binding cassette subfamily F member 3
OAS, 2'-5' oligoadenylate synthetase
OAS1B, 2'-5' oligoadenylate synthetase 1B
OAS1B-tr, truncated OAS1B in flavivirus-susceptible mice
OAS1BΔTM, truncated OAS1B without transmembrane domain
TNP-ATP, 2′(3′)-*O*-(2,4,6-Trinitrophenyl)adenosine 5′-triphosphate
MDR, multidrug resistance
HGT, horizontal gene transfer
NBD, nucleotide-binding domain
TMD, transmembrane domain
ARE, antibiotic resistance
WNV, West Nile virus
2-5A, 2'-5' linked oligoadenylates
ER, endoplasmic reticulum
ORP1L, oxysterol binding protein-related protein 1L
MEF, mouse embryonic fibroblast
BHK, baby hamster kidney cells
PAF, platelet-activating factor
LPC, lysophosphatidylcholine
LPI, lysophosphatidylinositol
Trp, tryptophan
NATA, *N*-Acetyl-L-tryptophanamide
P, pocket
IB, inclusion body
MGE, mobile genetic element

1 GENERAL INTRODUCTION

1.1 ABC Proteins and Multidrug Resistance

Multidrug resistance (MDR) is found in both cancer and bacterial cells. It results from development of resistance against multiple structurally unrelated drugs, which leads to treatment failure. Currently, MDR threatens human health worldwide with yearly estimates of 700,000 deaths from infectious bacteria and 600,000 deaths from resistance to chemotherapy drugs [1, 2]. By the year 2050, there are expected to be nearly 10 million annual deaths from MDR pathogens and about 8 million from MDR in cancer cells [1, 3]. Several mechanisms contribute to antibiotic resistance in bacterial cells. These include antibiotic efflux, antibiotic modification/degradation, antibiotic sequestration, and target modification/bypass/protection [4, 5]. Similar resistance mechanisms against chemotherapy drugs, such as drug efflux, drug inactivation, and drug target modifications, also occur in cancer cells [6, 7]. The most commonly seen mechanism for MDR in both bacterial and cancer cells results from the expression of efflux pump proteins belonging to different transporter protein families capable of transporting structurally diverse substrates. In bacteria, the efflux pump proteins belong to four families, which include the resistance-nodulation-division (RND) family, the major facilitator superfamily (MFS), the small multidrug resistance (SMR) family, the multidrug and toxic compound extrusion (MATE) family, and the ATP binding cassette (ABC) superfamily [8, 9]. In eukaryotic cells, drug efflux pumps typically belong to the ABC superfamily [6, 10]. Interestingly, most MDR proteins have also been shown to associate with and transport a variety of lipids, suggesting a common flipping mechanism for both types of molecules [11-17].

Of the protein families mentioned above, only ABC proteins power efflux by ATP hydrolysis which is coupled to substrate transport across cell membranes [18, 19]. All other

transporters use the energy of ion gradients [9]. Most ABC transporters are normally specific for either one molecule or a family of related compounds [18]. However, recently several promiscuous ABC proteins have been identified in both eukaryotic and prokaryotic organisms. These proteins are capable of transporting several structurally diverse compounds across cell membranes resulting in MDR in the cell. Human P-glycoprotein (Pgp, ABC subfamily B member 1, ABCB1) is a well-studied eukaryotic multidrug resistance exporter. Over-expression of Pgp in cancer cells leads to resistance to structurally diverse chemotherapeutic agents [6, 20, 21]. Breast cancer resistance protein (BCRP, ABC subfamily G member 2, ABCG2) is another eukaryotic ABC transporter whose over-expression in cancer cells leads to MDR development [6, 20]. Several prokaryotic organisms also contain polyspecific pumps evolutionarily-related to Pgp and other eukaryotic MDR pumps. For example, both DrrAB found in the antibiotic producer organism *Streptomyces peucetius* and the lipid A translocase MsbA in *Escherichia coli* confer MDR [11, 22, 23]. Due to the polyspecific nature of many bacterial exporters, an increasing concern is the mobilization of transporter pump genes from either antibiotic producer or environmental organisms to pathogenic bacteria by horizontal gene transfer (HGT) mechanisms [24, 25]. Different mechanisms for mobilization of antibiotic resistance genes, including MDR efflux pumps, to pathogenic organisms, are further discussed in the review in Chapter 2.

1.2 ABC Protein Structure and Classification

ABC transport proteins typically contain two nucleotide-binding domains (NBD) and two transmembrane domains (TMD) either located on separate subunits or in the same polypeptide chain [26]. The NBDs contain highly conserved sequence motifs, including the A-loop for ATP positioning, P-loop (Walker A) for ATP interactions, Walker B for ATP hydrolysis, the D-loop for catalytic site formation, H-loop (Switch) for Walker B motif and ATP positioning for catalysis,

the Q-loop for catalytic site formation when magnesium ions are present, and the ABC signature motif for ATP binding [26]. Of these, the best-studied motifs include the Walker A and Walker B motifs for ATP binding and hydrolysis, respectively [27]. The conserved lysine (K) residue of Walker A motifs interacts with the β- and γ-phosphates of ATP for nucleotide binding. Walker B motifs use a conserved glutamate (E) for ATP hydrolysis [26]. Recent crystal structure studies suggest that the two NBDs in ABC proteins associate with one another in a head-to-tail configuration. In this arrangement, the Walker A and Walker B motifs of one NBD associate with the ABC signature motif of the other NBD to form two ATP binding pockets at their interface [26, 28].

The ATP binding and hydrolysis performed by the conserved NBD motifs provides the energy for substrate transport by the TMDs [18, 29]. The substrate binding cavity is believed to be formed by helices contributed by different regions of the TMD, which may contain a similar three-dimensional structure in proteins belonging to the same transport family, although their sequences are not highly conserved [21, 26].

Based on their domain organization and function, ABC proteins are divided into three classes. Class 1 and class 3 ABC proteins contain both NBDs and TMDs, and they function as exporters and importers, respectively. Class 2 ABC proteins, however, only contain two fused NBDs. Due to the lack of TMDs, class 2 proteins have been suggested to be involved in regulatory functions [18]. Class 2 proteins are also further divided into three families: the RLI family for RNase L inhibitor proteins, ART family for antibiotic resistance and translation regulation, and the UVR family containing UvrA for DNA repair and DrrC for antibiotic resistance. The ART family is further subdivided into three subfamilies: the elongation factor 3 (EF3) translational

factor in yeast, the antibiotic resistance (ARE) subfamily, and the regulatory (REG) subfamily [18, 29].

According to the nomenclature for human ABC proteins, the ABC superfamily is organized into eight subfamilies (ABCA-ABCH) based on their structure and sequence homology. This terminology is also frequently utilized for all prokaryotic and eukaryotic ABC proteins [19]. In this designation, class 2 ABC proteins include the ABCE and ABCF subfamilies as they both lack TMDs and only contain two fused NBDs [18, 19].

Several ABCF proteins contain domains other than just NBDs. These domains include the Arm subdomain present in most bacterial ABCF proteins. Arms are located in the middle of the first NBD of bacterial ABCF proteins and have been shown to interact with the ribosomal protein L1 in the *E. coli* EttA protein [30, 31]. Most ABCF proteins also contain an inter-domain linker region fusing the two NBDs. In bacterial ABCF proteins, the linker domain interacts with the ribosome and is considered to be critical for function [31-33]. Other variations in ABCF protein structure include N- or C-terminal domain extensions [34]. The major focus of this dissertation is to elucidate the function of the mouse ABCF3 protein and its interaction with OAS1B, a flavivirus-resistance protein. Below, we describe the known functions of ABCF proteins, followed by a description of the ABCF3-OAS1B complex.

1.3 ABCF Proteins and Antibiotic Resistance

It has been previously suggested that ABCF proteins belonging to the ARE subfamily may associate with currently unidentified TMDs to form a complex for drug efflux from the cell in a mechanism similar to the other well-studied ABC efflux proteins, such as DrrAB and Pgp [35]. Currently, there is only one known example of an ABCF protein (MsrD) forming a complex with a TMD (the proton pump MefE) for the transport of macrolides in *E. coli* [36, 37].

Recent studies of other ARE ABCF proteins demonstrated that some of these proteins exert their functional roles through a ribosomal protection mechanism against drugs (macrolides, lincosamides, and streptogramins) that target the 50S ribosomal subunit [35]. For example, Vga(A) from *Staphylococcus hemolyticus* and LsaA from *Enterococcus faecalis* were demonstrated to have the ability to dislodge antibiotics from the peptidyl-transferase center of the ribosome using their linker domains [32, 33, 35]. Such proteins are of particular clinical importance as they are found in both antibiotic-producing bacteria (*Streptomyces*) and pathogenic bacteria [34], suggesting the possibility of the mobilization of these antibiotic resistant genes from producer organisms to clinical pathogens, which is further discussed in the review in Chapter 2.

1.4 ABCF Proteins and Translational Regulation

Other prokaryotic and eukaryotic ABCF proteins associate with the ribosome to perform translational regulatory functions, including translational initiation and elongation. Translation initiation factors include the mammalian ABC50 (ABCF1) and the yeast ARB1 (ABCF2) proteins involved in ribosome assembly [38-40]. Additionally, the *E. coli* protein Uup was recently found to associate with BipA for ribosome assembly, even though previous reports suggested Uup excised transposons [34, 41]. Regulatory ABCF proteins involved in translation elongation include the *E. coli* EttA protein which regulates entry into the translation elongation cycle during periods of starvation. Crystal structure analyses identified the linker domain of EttA as the P-site tRNA-interaction motif (PtIM) [30, 31]. Similar to EttA, the yeast GCN20 (ABCF3) protein also regulates translation elongation during amino-acid starvation conditions [42, 43]. Finally, the yeast EF3 protein is also known to be involved in polypeptide elongation and ribosome recycling [44-47].

1.5 Other Known Functions of ABCF Proteins

In addition to the antibiotic resistance and translational regulation described above, ABCF proteins have also been shown to affect other diverse cellular functions. For example, several studies have reported an enhanced expression of human ABCF2 in ovarian cancer, and one study observed an association of human ABCF3 with the tumor-inducing protein TPD52L2 that led to the proliferation of liver cancer cells [48-50]. Other studies on human ABCF2 suggested it regulates the volume-sensitive outwardly rectifying anion channel (VOSR) to provide an anti-apoptotic effect [51]. ABC50 (ABCF1), in addition to regulating translation initiation, also has a role in innate immunity against retroviruses as well as in the promotion of phagocytosis of retinal pigment epithelial cells [38, 39, 52, 53]. A study on the *Caenorhabditis elegans* ABCF3 protein demonstrated its function as a translation initiation factor with GCN-1 to promote apoptosis [54]. Recently, the mouse ABCF3 protein was shown to contribute to the flavivirus resistance provided by its partner protein OAS1B [55]. Biochemical analysis of mouse ABCF3 and expression of the ABCF3-OAS1B complex in bacteria form the focus of Chapter 1 in this dissertation.

1.6 ABCF3-OAS1B and Flavivirus Resistance

The genus *Flavivirus* includes West Nile virus (WNV), dengue virus, yellow fever virus, and Zika virus. WNV was previously endemic to Africa, Australia, and parts of Asia, but since its arrival in North America in 1999, the virus has spread and is now endemic throughout the United States [56]. Since its introduction to the USA, WNV has infected over 45,000 people, causing over 1500 deaths [56]. Both globalization and a warming climate are expected to further increase the spread of this virus to new geographical areas leading to enhanced numbers of infections. Natural viral transmission occurs between mosquitoes and birds; however, both horses and humans may be infected through mosquito bites [56]. In most cases of human infection, there are no symptoms,

or they appear to be flu-like. However, nearly 25% of infections result in fever or other symptoms, with 1 in 150 developing a neuroinvasive disease (meningitis, encephalitis, or paralysis) that may be fatal. The elderly and immunocompromised are the most susceptible to these neuroinvasive diseases and have an increased risk of infection-related death. To date, there is no human vaccine or antiviral drug against WNV, and treatment only alleviates symptoms [57, 58].

Flavivirus resistance was mapped to the *Flv* locus in resistant mice that encodes for the 2'-5'oligoadenylate synthetase (OAS) protein 1B [59]. OAS family proteins provide innate immunity in host cells against viral infections, including WNV [58]. Typical OAS proteins generate 2'-5' linked oligoadenylates (2-5A) in response to binding to dsRNA [60, 61]. 2-5A activation of RNase L leads to its dimerization, and activated RNase L degrades single-stranded cellular and viral RNA [62]. Mouse OAS1B, however, is an inactive synthetase with antiviral activity unrelated to the OAS/RNase L activation pathway for RNA degradation [63]. The resistance allele encodes the full-length OAS1B, whose C-terminal TMD locates the protein to the endoplasmic reticulum (ER) membrane [55, 64]. A premature stop codon in the susceptible allele produces a truncated version of OAS1B (OAS1B-tr) that lacks the C-terminal TMD [59, 64]. The absence of the TMD prevents its localization to the ER membrane. A recent study identified two binding partners for OAS1B: ABC subfamily F member 3 (ABCF3) and oxysterol binding protein-related protein 1L (ORP1L), which most likely form a complex with OAS1B to enhance antiviral function [55].

1.7 ABCF3-OAS1B-ORP1L Complex

Initially, ABCF3 and ORP1L were discovered as binding partners for the mouse OAS1B protein in a yeast-two hybrid screen using a mouse brain library [55]. OAS1B-ABCF3 and OAS1B-ORP1L interactions were further confirmed with an *in vitro* co-immunoprecipitation assay using both full-length OAS1B and truncated OAS1B-tr. ABCF3 co-precipitated with both

full-length and truncated OAS1B-tr, whereas ORP1L co-precipitated with only full-length OAS1B. These results suggested that ABCF3 interacts with a region of OAS1B N-terminal of its premature stop codon, and ORP1L interacts with OAS1B C-terminal of the premature stop codon. Further confirmation of both OAS1B-ABCF3 and OAS1B-ORP1L interactions was obtained by an *in vivo* co-immunoprecipitation assay using mouse embryonic fibroblasts (MEFs) either mock-infected or infected with WNV. In this assay, not only did both ABCF3 and ORP1L individually co-precipitate with OAS1B with or without WNV infection, but the ABCF3-OAS1B-ORP1L complex also co-precipitated with the NS3 helicase of WNV from infected cells, a viral protein that is a member of the viral replication complex located on the ER membrane. These results suggested that a complex of ABCF3-OAS1B-ORP1L may interact with WNV replication complexes at the ER; however, co-precipitation of the NS3 protein does not identify a viral protein partner since antibodies to other nonstructural proteins were not tested. Finally, the co-localization of these proteins was analyzed *in vivo* after co-transfection of *oas1b* cDNA with either *abcf3* or *orp1l* cDNA in mock-infected or WNV infected baby hamster kidney (BHK) cells using an immunofluorescence assay (IFA). In both mock and WNV infected BHK cells, OAS1B co-localized with ABCF3 and ORP1L [55].

ABCF3 knockdown in MEFs resulted in enhanced levels of the flavivirus WNV but not that of members of other viral families, suggesting that ABCF3 contributes to the OAS1B-mediated specific flavivirus resistance mechanism. Additionally, lack of ABCF3 had an effect on the flavivirus resistance mechanism only when full-length OAS1B was expressed in MEFs, confirming that localization of the complex to the ER membrane is essential for antiviral function to occur. Knockdown of ORP1L, on the other hand, led to decreased viral titers of WNV as well as of the non-flaviviruses Sindbis virus (SINV) and vesicular stomatitis virus (VSV), leading to

speculation that knockdown of ORP1L dysregulates late endosome function and endosomes are required for cell entry by all of these viruses, therefore the effect of ORP1L knockdown on viral entry prevents the use of this strategy for studying the contribution of ORP1L to the flavivirus resistance phenotype [55].

1.8 Biochemical Characterization of mouse ABCF3

To gain an understanding of the contribution of ABCF3 to the flavivirus resistance mechanism, we biochemically characterized the mouse ABCF3 protein by examination of its ATP binding and hydrolysis activities in Chapter 1. Initially, we tested multiple bacterial systems (pE-SUMO, pGEX-6p-1, and pET28a) for *abcf3* expression and purification, with the His-tag pET28a vector providing the best results. After optimization of ABCF3 purification from this vector, we demonstrated that purified ABCF3 contains a basal ATPase activity of around 130 nmol/min/mg. We then used the fluorescent ATP analog, TNP-ATP, to examine the nucleotide-binding properties of ABCF3. Mutation analyses combined with TNP-ATP binding analyses showed that ABCF3 contains two asymmetric NBDs that appear to have different functions. To elucidate possible substrates of ABCF3, we tested the effect of several drugs and lipids. Many MDR ABC proteins are known to associate with and transport both drugs (Hoechst 33342, verapamil, vinblastine, and quinidine) and lipids (fatty acids, sterols, phospholipids, and sphingolipids) [11, 13, 15-17, 22, 65-67]. For example, the *E. coli* MsbA protein was previously shown to transport several MDR drugs although the physiological function of MsbA is to flip lipid A from the inner to the outer leaflet of the cytoplasmic membrane [11, 12]. Another well-studied human ABC MDR protein, Pgp, with well-known polyspecific drug efflux activity, also demonstrated lipid flippase activity for several phospholipid species [14, 21]. Thus, in this study, we tested the effects of MDR drugs, such as quinidine, verapamil, vinblastine, and Hoescht 33342, on the activity of purified ABCF3. None of

the tested MDR drugs either stimulated or inhibited ABCF3 ATPase activity. The OAS1B-ABCF3 interaction provides flavivirus resistance only when located at the ER membrane, the site of flavivirus replication and lipid biosynthesis [55, 68]. Lipid biosynthesis is increased by flavivirus infection [69]. Since flavivirus infections induce lipid reorganization in the ER and modulation of host cell lipid metabolism [69], we also tested the effect of various lipids on the ATPase activity of purified ABCF3 in Chapter 1. We show that some lipids, including sphingosine, sphingomyelin, platelet-activating factor (PAF), and lysophosphatidylcholine (LPC) stimulated ABCF3 ATPase activity, while cholesterol and some lipid-based drugs, such as miltefosine, perifosine, and edelfosine, either inhibited or produced a biphasic response. Interestingly, ABCF3 proteins with NBD1 and NBD2 mutations exhibited differential effects on both basal and sphingosine-stimulated ATPase activities. Based on the observations described above, a model for the function of ABCF3 is presented in Chapter 1.

1.9 ABCF3 and OAS1B Interaction

ABCF3 and OAS1B interaction has been previously indicated by a yeast-two hybrid screen, by *in vitro* co-immunoprecipitation assays, and by *in vivo* co-immunoprecipitation assays. Moreover, co-localization of the two proteins was detected by IFA in mammalian cells [55], as described in Section 1.7 above. In this dissertation, we analyzed the interaction between these two proteins in bacterial cells. The pETDuet-1 vector, previously used to examine protein complexes in *E. coli* cells [70-72], was used for simultaneous co-expression of OAS1B and ABCF3. Interestingly, the simultaneous expression of the two proteins in bacteria provided strong, though indirect, evidence for interaction between ABCF3 and OAS1B, through both a growth rescue phenotype and by stabilization of large amounts of OAS1B in bacterial cells. However, as shown in Chapter 1, OAS1B protein strongly localized to inclusion bodies in *E. coli* cells, even when *E.*

coli FtsH transmembrane domains were fused to truncated OAS1B proteins to promote membrane localization. Future applications of the bacterial co-expression system for identifying the domains of ABCF3 involved in interaction with OAS1B and in providing an additional system for biochemical analysis of these proteins are described in Chapter 1. Possible mechanisms for the role of ABCF3 in OAS1B-mediated flavivirus resistance are also discussed later.

2 CHAPTER I: BIOCHEMICAL CHARACTERIZATION OF THE MOUSE ABCF3 PROTEIN, A PARTNER OF THE FLAVIVIRUS-RESISTANCE PROTEIN OAS1B

Mammalian ATP-binding cassette subfamily F member 3 (ABCF3) is a class 2 ABC protein that has previously been identified as a partner of the mouse flavivirus resistance protein 2′,5′-oligoadenylate synthetase 1B (OAS1B). The functions and natural substrates of ABCF3 are not known. In this study, analysis of purified ABCF3 showed that it is an active ATPase, and binding analyses with a fluorescent ATP analog suggested unequal contributions by the two nucleotide-binding domains. We further showed that ABCF3 activity is increased by lipids, including sphingosine, sphingomyelin, platelet-activating factor, and lysophosphatidylcholine. However, cholesterol inhibited ABCF3 activity, whereas alkyl ether lipids either inhibited or resulted in a biphasic response, suggesting small changes in lipid structure differentially affect ABCF3 activity. Point mutations in the two nucleotide-binding domains of ABCF3 affected sphingosine-stimulated ATPase activity differently, further supporting different roles for the two catalytic pockets. We propose a model in which pocket 1 is the site of basal catalysis, whereas pocket 2 engages in ligand-stimulated ATP hydrolysis. Co-localization of the ABCF3–OAS1B complex to the virus-remodeled endoplasmic reticulum membrane has been shown before. We also noted that co-expression of ABCF3 and OAS1B in bacteria alleviated growth inhibition caused by expression of OAS1B alone, and ABCF3 significantly enhanced OAS1B levels, indirectly showing interaction between these two proteins in bacterial cells. As viral RNA synthesis requires large amounts of ATP, we conclude that lipid-stimulated ATP hydrolysis may contribute to the reduction in viral RNA production characteristic of the flavivirus resistance phenotype.

2.1 Introduction

Members of the genus *Flavivirus* in the family Flaviviridae include human pathogens, such as West Nile virus (WNV), Japanese encephalitis virus, tick-borne encephalitis virus, yellow fever virus, dengue virus, and Zika virus. Phenotypic evidence of genetically controlled host resistance to particular virus pathogens has previously been obtained but few of the genes involved have been identified and characterized (1). Flavivirus resistance and susceptibility in mice is controlled by the alleles of the *Flv* locus, which encode the 2'-5'-oligoadenylate synthetase (OAS) 1B protein. Flavivirus-resistant mice express a full-length OAS1B protein, while susceptible mice produce a truncated protein (OAS1B-tr) due to the presence of a premature stop codon (2,3). *oas1* genes are components of the cellular innate immune response that when activated by viral dsRNA synthesize short 2'-5'-linked oligoadenylates (2-5A). These bind to cytoplasmic RNase L causing it to dimerize and cleave single-stranded cell and viral RNAs (4). Eight orthologs of the *oas1* gene (*oas1a-h*) have been identified in mice (5,6). The proteins produced by only two of the murine *oas1* genes (OAS1A and OAS1G) are active synthetases. OAS1B is an inactive synthetase that cannot produce 2-5A (7,8).

The OAS1B protein localizes to the endoplasmic reticulum (ER) through a C-terminal transmembrane domain consisting of 23 amino acid residues (9). OAS1B-tr, the truncated version of OAS1B, lacks this C-terminal transmembrane domain and is therefore unable to anchor to the ER. Flavivirus RNA replication occurs within invaginations in the ER membrane (10). Although flaviviruses can attach and enter resistant and susceptible mouse cells with similar efficiency, resistant cells produce reduced levels of intracellular flavivirus RNA as well as lower virus yields (9). A yeast two-hybrid screen of a mouse brain library identified two binding partners for OAS1B: ABCF3 which belongs to class 2 of the ATP-binding cassette (ABC) superfamily of proteins; and

ORP1L, a protein involved in sterol binding and regulation of late endosome motility as well as protein and lipid transport (9,11). Interaction between OAS1B and ABCF3 was further demonstrated by co-immunoprecipitation in mammalian lysates and co-localization in baby hamster kidney cells by fluorescence microscopy (9). Knockdown of ABCF3 protein levels increased WNV yields but not those of two nonflaviviruses, vesicular stomatitis virus and Sindbis virus, supporting a specific role for ABCF3 in OAS1B-mediated flavivirus resistance (9). Moreover, the flavivirus-specific effect of knockdown of ABCF3 was only seen in resistant mouse embryo fibroblasts (MEFs) that naturally express the full-length OAS1B protein and not in susceptible MEFs expressing the truncated OAS1B-tr (9).

Most ABC proteins contain two nucleotide-binding domains (NBDs) and two transmembrane domains (TMDs) that are present either in the same polypeptide or on separate subunits. The NBDs contain several conserved motifs, including Walker A, Walker B, ABC Signature, Q-loop, and Switch motifs, and the TMDs have limited sequence conservation (12). The Walker A motif of ABC proteins plays a critical role in ATP binding, and the Walker B is involved in hydrolysis (12). Analyses of the crystal structures of ABC proteins suggest that their ATP-binding pockets are located at the interface formed by the Walker A motif of one NBD juxtaposed against the signature motif of the other NBD in a head-to-tail configuration (12,13). ABC proteins are normally involved in the cellular transport of a diverse range of substrates in both prokaryotes and eukaryotes, and this process is coupled to the energy of ATP hydrolysis (14,15). Members of the ABC superfamily are divided into three classes (14,15). Although the function, mechanism, and structure of class 1 and class 3 proteins have been elucidated in detail (14,15), little is known about class 2 proteins and their physiological roles. Class 2 proteins are distinct in that they lack the TMD domains but contain two tandemly-linked NBD domains, which

also likely participate in a head-to-tail configuration resulting in two ATP binding pockets (13). Because of the lack of a transmembrane domain, the primary cellular role of most class 2 proteins is believed to be regulatory in nature (15). It has been postulated that some class 2 proteins may complex with other cellular membrane proteins to enable them to transport ligands. The bacterial Mel protein that interacts with the proton-motive force-driven transmembrane pump protein MefE to form a complex involved in the transport of erythromycin is the single example of such a complex available to date (16,17).

The ABC proteins have also been assigned to 8 subfamilies, A to H. Subfamilies E and F, which do not contain TMDs, belong to ABC class 2 (15). Subgroup F includes mammalian F1, F2, and F3; yeast GCN20 and EF3; and bacterial EttA, Vga(A), and Uup proteins. Some bacterial ABCF proteins, such as Vga(A), confer antibiotic resistance by drug displacement at the peptidyl transferase center of the ribosome (18-22). EttA, which functions as a translation factor and regulates progression of the 70S initiation complexes into the elongation cycle, also associates with the ribosome (23,24). Association with the ribosome was also reported for other ABCF proteins, including mammalian ABCF1 (ABC50) and yeast EF3, ARB1 (ABCF2), and GCN20 (ABCF3) proteins, which regulate protein translation either at the level of initiation or elongation (25-33). Additional reports suggesting that the eukaryotic F1, F2, and F3 proteins can impact diverse cellular activities, including innate immunity against retroviruses (34), promotion of phagocytosis (35), anti-apoptotic effect (36), and co-localization with a tumor-inducing protein (37) are also available; however there is no firm consensus about their cellular functions, and none have been characterized biochemically. However, data from limited biochemical analyses of the bacterial ABCF proteins Vga(A), Uup, and EttA are available. All three of these bacterial ABCF proteins have ATPase activity, which was shown to be essential for their function in the cell

(23,38,39). The ATPase activity of Vga(A) was found to be inhibited by the antibiotic pristinamycin IIA (38), suggesting direct binding of Vga(A) with its ligand, although there is no known partner protein with a TMD. By contrast, ABC proteins belonging to class 1 and 3 normally bind their ligands only when in complex with their cognate TMD partner proteins (14). Finally, the bacterial ABCF proteins also have about an 80-amino acid long inter-ABC domain linker, which contains conserved sequences and is rich in positively-charged residues (40). In the case of Vga(A) and EttA, the linker region was shown, by mutagenesis and deletion analysis, to be critical for their association with the ribosome and for their function (20,21,23). Interestingly, association of the EttA linker with the ribosome was found to be sensitive to the ATP/ADP ratio, leading to the proposal that this protein plays a role in regulation of protein chain elongation in energy-starved cells (23).

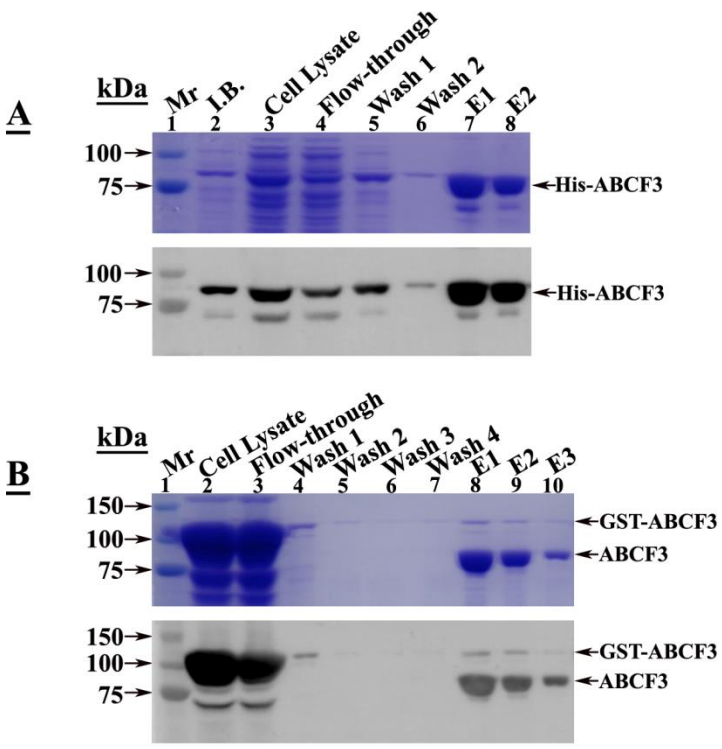
To gain an understanding of mammalian ABCF3 protein functions that may play a role in the OAS1B-mediated flavivirus-resistance mechanism, the ATP-binding and ATPase activities of mouse ABCF3 were characterized, potential ligands of ABCF3 were identified, and the ability of ABCF3 to interact with OAS1B in bacterial cells was analyzed. We showed that purified ABCF3 protein is an active ATPase with both NBDs contributing to the catalytic activity. TNP-ATP binding studies showed that the two NBDs of ABCF3 are asymmetric with NBD2 playing a more important role in nucleotide binding. The substrates of the ABCF3 protein are currently not known. However, many ABC family proteins are known to transport lipids and amphiphilic drugs, and their ATPase activities have been shown to be stimulated or inhibited by these substrates (41-49). Moreover, flavivirus infections modulate host cell lipid metabolism (50) and result in changes in the levels of fatty acids, phospholipids, sphingolipids, and cholesterol in cell membranes (51-53), including in the ER which is the site of OAS1B/ABCF3 localization and a major site for lipid

biosynthesis (9,54). Therefore, in this study we tested the effect of multiple lipids and amphiphilic drugs on ABCF3 activity. Interestingly, the ATPase activity of ABCF3 was found to be modulated by several of the tested lipids, but not by amphiphilic drugs. The basal and lipid-stimulated ATPase activity data obtained with ABCF3 mutated in NBD1 and NBD2 suggested that the two ATP binding pockets may play different roles in ATP hydrolysis. Co-expression of *abcf3* and *oas1b* in bacteria resulted in alleviation of growth inhibition caused by *oas1b* expression alone and significantly enhanced OAS1B levels, suggesting an intracellular protein-protein interaction in bacterial cells.

2.2 Results

2.2.1 Analysis of the ATPase activity of ABCF3

ABCF3 protein was expressed from pET28a-*abcf3* (Fig. 2.1A) or pGEX-*abcf3* (Fig. 2.1B), as described under "Experimental procedures." These two clones produced ABCF3 with an N-terminal His-tag and GST-tag, respectively. A basal activity of 39 nmol/min/mg in 50 mM HEPES containing 125 mM NaCl, pH 7.5, was observed when the GST-tag was still present (Fig. 2.1, B and C). In contrast, a basal activity of 125 nmol/min/mg was observed when the GST-tag was removed. His-tagged ABCF3 exhibited a basal ATPase activity of 132 nmol/min/mg that was similar to that of untagged ABCF3 (Fig. 2.1, A and C), indicating that the presence of the His-tag at the N terminus of ABCF3 had no deleterious effect on its activity. Also, the yield of pGEX*f3*-expressed ABCF3 protein obtained after removal of the GST tag was significantly lower than that of pET28a*f3*-expressed His-ABCF3 (Fig. 2.1, A-C). Purified His-tagged ABCF3 protein was used in the subsequent experiments.



C

ATPase Activity of Purified ABCF3		
Protein	Yield (mg/ml)	ATPase Activity (nmole/min/mg)
GST-ABCF3	1.3	39 ± 12
ABCF3 (no GST Tag)	0.4	125 ± 6
His-ABCF3	1.3	132 ± 20

Figure 2.1: Expression and purification of ABCF3.

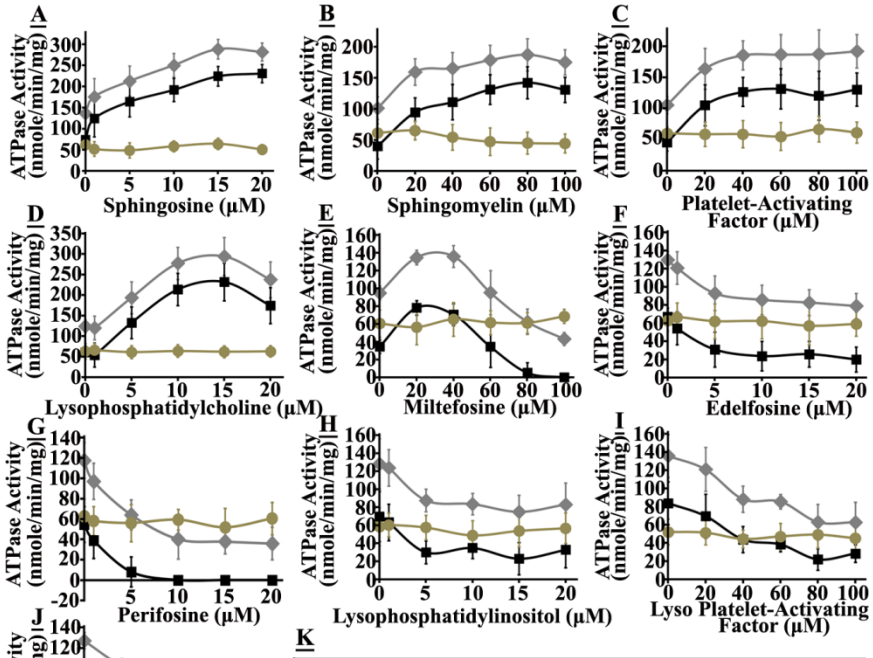
Protein purification and ATPase activity assays were performed as described under "Experimental procedures." *A, E. coli* HMS174(DE3) cells containing pET28a-*abcf3* were used for purification of ABCF3. Samples 2-6 were normalized by volume. Equal volumes (10 µl) were then analyzed using 10% SDS-PAGE, followed by staining with Coomassie Blue and Western blot analysis using anti-ABCF3 (1:2000). Two µl of the 1-ml fractions were analyzed in *lanes 7* and *8*. *Lane 1*, Mr (marker); *lane 2* (I.B.) inclusion body; *lane 3*, cell lysate; *lane 4*, column flow-through; *lane 5*, washed with 50 ml of 30 mM imidazole in Buffer A (1x PBS, 20% glycerol, pH 7.4); *lane 6*, washed with 1 ml of 100 mM imidazole in Buffer A; *lanes 7* and *8*, 1 ml of the elution fractions, each with 200 mM imidazole in Buffer A. *B, E. coli* Rosetta 2(DE3)pLysS cells containing pGEX-*abcf3* were used for purification of ABCF3 with GST-tag cleavage during elution. Samples 2-7 were normalized by volume. Equal volumes (10 µl) were then analyzed using 10% SDS-PAGE, followed by staining with Coomassie Blue and Western blot analysis using anti-ABCF3 (1:2000).

Two µl of 1-ml elution were analyzed in *lanes 8-10*. *Lane 1*, Mr; *lane 2*, cell lysate; *lane 3*, column flow-through; *lanes 4-6*, washed with 10 ml of Buffer A each; *lane 7*, washed with 20 ml Buffer B (1x cleavage buffer, 20% glycerol, pH 7.0); *lanes 8-10*, elution fractions 1-3 with 1 ml of Buffer B after GST tag cleavage with PreScission Protease (GE Healthcare). *C, table* shows the yield of purified ABCF3 and mean ± S.D. n=10 experiments for 5 µg basal ABCF3 ATPase activity. A Mann-Whitney test showed there was a significant difference between basal ATPase activity of GST-ABCF3 and ABCF3, *p* value 0.0022; between GST-ABCF3 and His-ABCF3, *p* value 0.0007; and there was no significant difference between ABCF3 and His-ABCF3 basal ATPase activities, *p* value 0.7546. The pGEX-*abcf3* clone was created by Chao Zhao.

2.2.2 Modulation of the ATPase activity of ABCF3 by potential ligands

The substrates of the ABCF3 protein are currently unknown. To identify potential ligands, the effect of various lipids, sterols, and drugs on the ATPase activity of ABCF3 was analyzed (Fig. 2.2, A-J, *gray diamond*) as described under "Experimental procedures." The selected lipids and drugs were chosen based on previous reports in the literature (11,45-49). To remove background activity, the effect of each ligand on the activity of the double Walker A mutant K216A/K531A (described later) was also analyzed (Fig. 2.2, A-J, *tan circle*). The normalized activities were then calculated by subtracting the activity of the double mutant from the WT ABCF3 activity at each ligand concentration (Fig. 2.2, A-J, *black square*). The data in Fig. 2.2 show that the ATPase activity of ABCF3 was significantly modulated by sphingosine, sphingomyelin, platelet-activating factor (PAF), lysophosphatidylcholine (LPC), lysophosophatidylinositol (LPI), lyso-PAF, cholesterol, and alkyl ether lipids. Sphingosine, sphingomyelin, PAF, and LPC induced nearly a 3-fold enhancement in activity (Fig. 2.2, A-D). The alkyl ether lipid miltefosine induced a biphasic

response with ATPase activity stimulated at low concentrations and inhibited at higher concentrations (Fig. 2.2E). In contrast, the other two alkyl lipids, edelfosine and perifosine, as well as LPI, lyso-PAF, and cholesterol, inhibited ATPase activity at all concentrations tested, including very low concentrations (Fig. 2.2, F-J). Several drugs, which are known to be substrates of multidrug resistance pumps, such as Hoechst 33342, verapamil, vinblastine, and quinidine as well as lipids, including phosphatidylcholine, phosphatidylethanolamine, sphingosine-1-phosphate, ceramide and dihydroceramide had no effect on the ATPase activity of ABCF3 (Fig. 2.2K).



K

Effect of Potential Ligands on ABCF3 ATPase Activity	
MDR Drugs	**Fold Change**
Hoechst 33342	No Effect
Quinidine	No Effect
Verapamil	No Effect
Vinblastine	No Effect
Lipids, Sterol	**Fold Change**
Sphingosine	2.92 (15μM**)
Sphingosine-1-Phosphate	No Effect
Sphingomyelin	3.08 (100μM*)
Ceramide	No Effect
Dihydroceramide	No Effect
Platelet-activating factor	2.80 (40μM*)
Lyso Platelet-activating factor	0.26 (80μM*)
10:0 Phosphatidylcholine	No Effect
14:0 Phosphatidylethanolamine	No Effect
Lysophosphatidylcholine	3.83 (15μM***)
Lysophosphatidylinositol	0.33 (15μM***)
Cholesterol	0.32 (80μM*)
Alkyl Ether Lipids	**Fold Change**
Miltefosine	2.05 (40μM*) ; 0.15 (80μM*)
Edelfosine	0.30 (20μM**)
Perifosine	0.13 (5μM*)

Figure 2.2: Modulation of the ATPase activity of purified ABCF3 by ligands.

A-J, increasing concentrations of sphingosine *(A)*, sphingomyelin *(B)*, platelet-activating factor *(C)*, lysophosphatidlycholine *(D)*, miltefosine *(E)*, edelfosine *(F)*, perifosine *(G)*, lysophosphatidylinositol *(H)*, lyso platelet-activating factor *(I)*, and cholesterol *(J)* were added to 5 μg of purified ABCF3 in a 1-ml volume reaction. The coupled assay for ATPase activity was carried out, as described under "Experimental procedures." Data points represent mean ATPase activity with standard deviation for 10 trials in nanomoles/min/mg. *Gray diamond* indicates WT

ABCF3; *tan circle* indicates K216A/K531A mutant ABCF3; and *black square* indicates normalized WT ABCF3 ATPase activities. ATPase activities were normalized by subtracting the activity of K216A/K531A mutant protein from WT ABCF3 activity at each ligand concentration. *K, table* shows a summary of fold-change in ATPase activity produced by various ligands. The activity in the absence of a ligand was designated as 1.0. Fold change > 1 implies stimulation, and < 1 implies inhibition. The reported fold-change was observed at the concentration indicated in *parentheses*. The results of Wilcoxon matched-pairs signed rank tests comparing normalized basal ATPase activity and normalized ligand-stimulated activity of WT ABCF3 are shown next to the ligand concentration values. *** p value ≤ 0.001; ** p value ≤ 0.01; * p value ≤ 0.05. Part of the data in this figure was obtained by Emma Shippee.

2.2.3 Role of the two NBDs of ABCF3 in ATP binding

Similar to other class 2 ABC proteins, mouse ABCF3 contains two tandem NBDs connected by an 80-amino acid long linker sequence with each NBD containing all of the previously described conserved motifs (Fig. 2.3A). Alignment of the amino acid sequence of mouse ABCF3 with those of other class 2 eukaryotic and prokaryotic ABC proteins showed a very high degree of sequence similarity in all of the conserved motifs present in both NBD1 (Fig. 2.3B) and NBD2 (Fig. 2.3C). The human and mouse ABCF3 proteins showed more than 95% overall sequence identity with each other extending over the entire sequence of these proteins. The inter-ABC domain linker region of bacterial ABCF proteins contains conserved sequences and several charged residues (Fig. 2.4A) (21,23,40). An alignment of the bacterial and eukaryotic linker sequences showed regions of relatively high homology between the two groups (marked with *green highlighted boxes*, Fig. 2.4B) and within members of the eukaryotic group (Fig. 2.4C),

suggesting that the linker region of eukaryotic proteins may also play an important role in the function of these proteins.

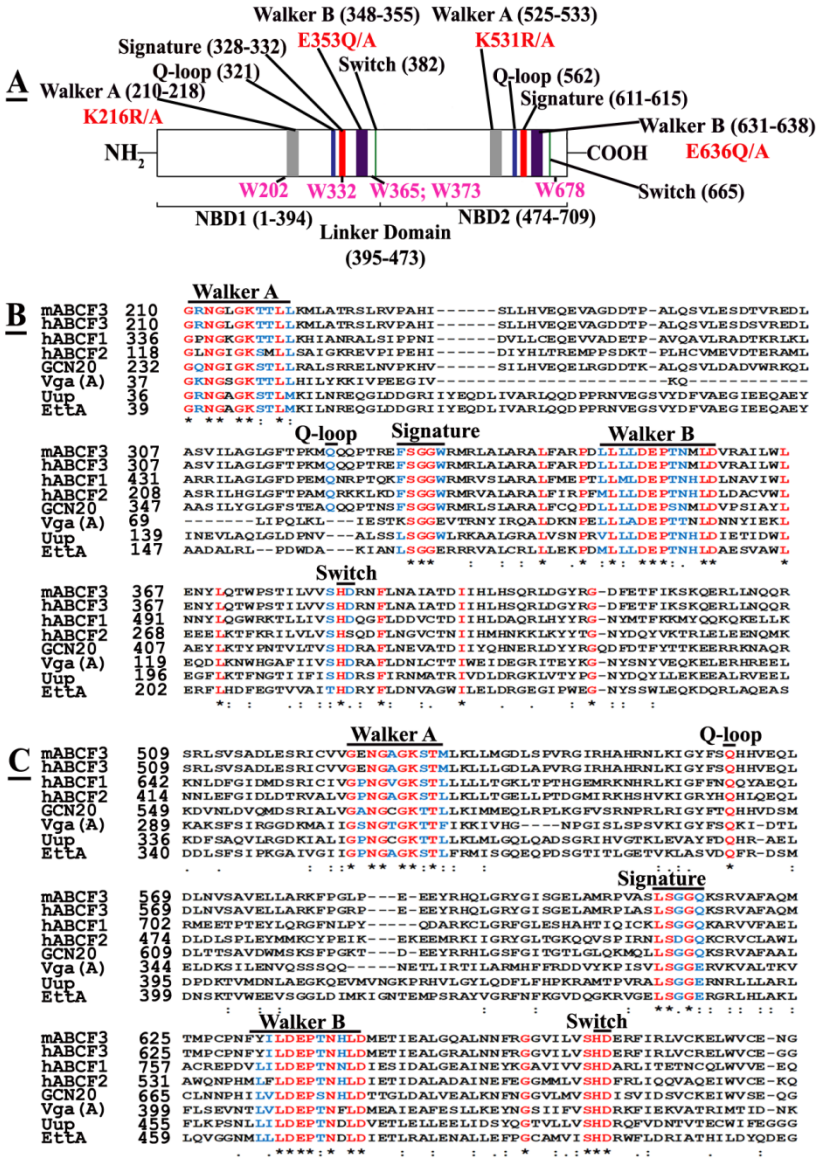


Figure 2.3: ABCF3 schematic and alignment.

A, schematic representation of conserved domains in the mouse ABCF3 protein, with numbers indicating the location of specific amino acid residues. Tryptophan residues are shown in *pink* (W202, W332, W365, W373, W678) and the mutated Walker A and Walker B residues in *red font*.

B and C, Clustal Omega sequence alignment of NBD1 *(B)* and NBD2 *(C)* of mouse ABCF3 with selected class 2 eukaryotic and prokaryotic ABC proteins. *Red*, identical residues; *blue*, similar residues. The PDB accession number and source of each protein is as follows: mABCF3 (NP_038880.1) *Mus musculus*; hABCF3 (AIC56674.1), hABCF1 (AQY76225.1), and hABCF2 (NP_009120.1) *Homo sampiens*; GCN20 (KZV11610.1) *Saccharomyces cerevisiae*; Vga(A) (AAA26684.1) *Staphylococcus aureus*; and Uup (P43672.2); and EttA (P0A9W3.2), *Escherichia coli*.

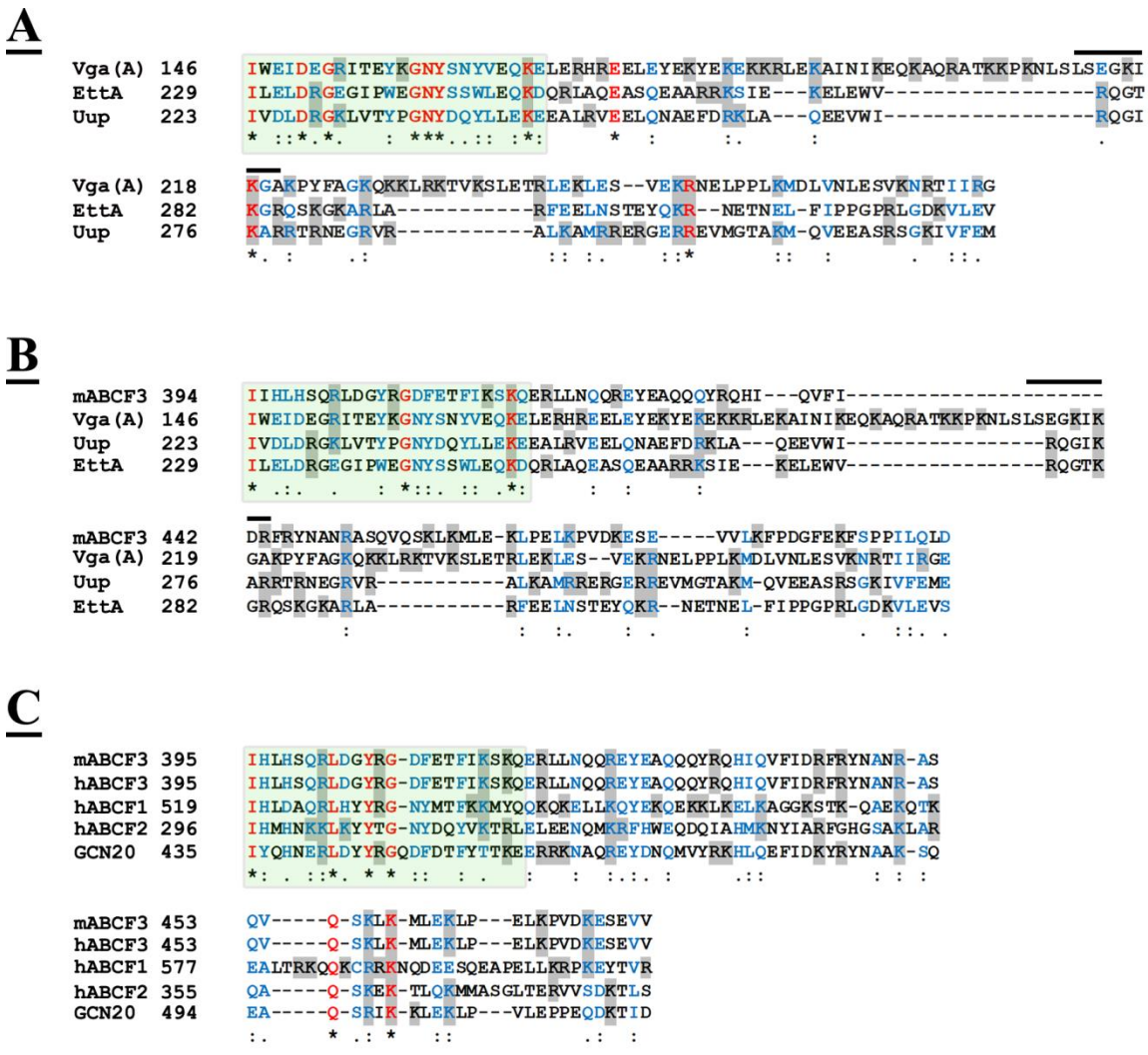
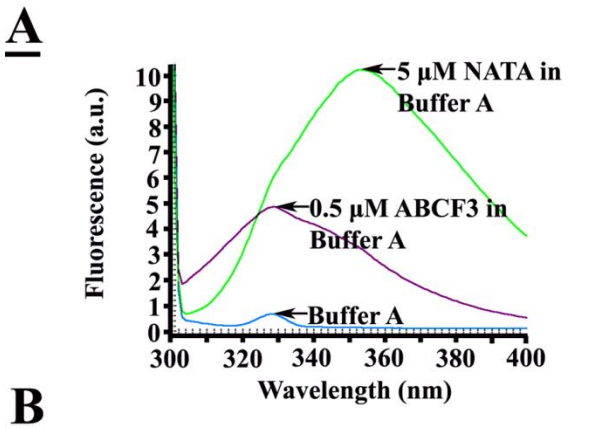


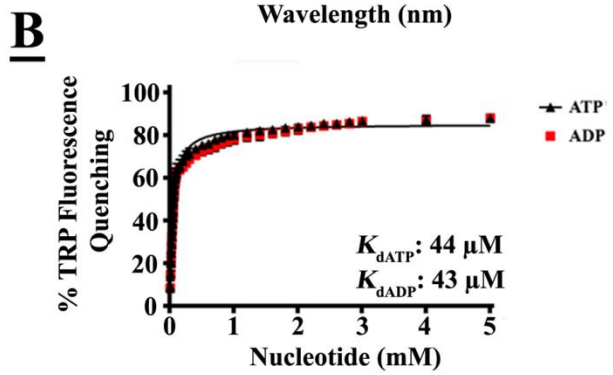
Figure 2.4: Inter-domain linker alignment of ABCF proteins.

A-C, Clustal Omega sequence alignment of inter-domain linker of selected class 2 prokaryotic *(A)* ABC proteins; mouse ABCF3 with class 2 prokaryotic *(B)* ABC proteins; and mouse ABCF3 with class 2 eukaryotic *(C)* ABC proteins. *Red*, identical residues; *blue*, similar residues. Positively charged residues lysine (K) and arginine (R) are *highlighted* in *gray*. The *green highlighted boxes* indicate regions of high homology. A region of Vga(A) (212-220) previously shown to be important for antibiotic resistance is denoted with a *black line*. The PDB accession number and source of each protein is as follows: mABCF3 (NP_038880.1) *Mus musculus*; hABCF3 (AIC56674.1), hABCF1 (AQY76225.1), and hABCF2 (NP_009120.1) *Homo sampiens*; GCN20 (KZV11610.1) *Saccharomyces cerevisiae*; Vga(A) (AAA26684.1) *Staphylococcus aureus*; and Uup (P43672.2); and EttA (P0A9W3.2), *Escherichia coli*.

To determine the function of each NBD of the mouse ABCF3 protein, point mutations were made in either the conserved lysine in the Walker A motif that is known to be critical for ATP binding or in the conserved glutamate in the Walker B motif that plays an important role in ATP hydrolysis (12,14). Clones containing simultaneous mutations in both NBDs of ABCF3 were also made. The nucleotide-binding characteristics of the purified WT and mutated ABCF3 proteins were initially analyzed by intrinsic tryptophan (Trp) fluorescence quenching. This approach is commonly used to determine conformational changes in proteins in response to the binding of nucleotides and other substrates (42). An emission scan of ABCF3 and NATA (a tryptophan analog) showed that, as expected, the environment of the Trp residues in ABCF3 is more nonpolar than that of NATA (Fig. 2.5A). Titration of purified ABCF3 protein with ATP or ADP showed saturable quenching, indicating specific binding of each nucleotide (Fig. 2.5B). However, the corrected fluorescence data could be fitted to single-site Michaelis-Menten kinetics suggesting that

there is only one nucleotide binding site in WT ABCF3 (Fig. 2.5B). This may be due to the asymmetric distribution of the Trp residues in ABCF3 (with four located in NBD1 and only one near NBD2, Fig. 2.3A), which likely introduces a bias in the Trp-quenching experiments. Analysis of single or double Walker A mutants (K216R, K531R, or K216R/K531R) surprisingly showed that the ATP-binding affinity of each of these mutants was unaffected compared with that of WT ABCF3 (Fig. 2.5C), further indicating that Trp-quenching analysis may not be suitable for studying nucleotide binding to ABCF3.





C

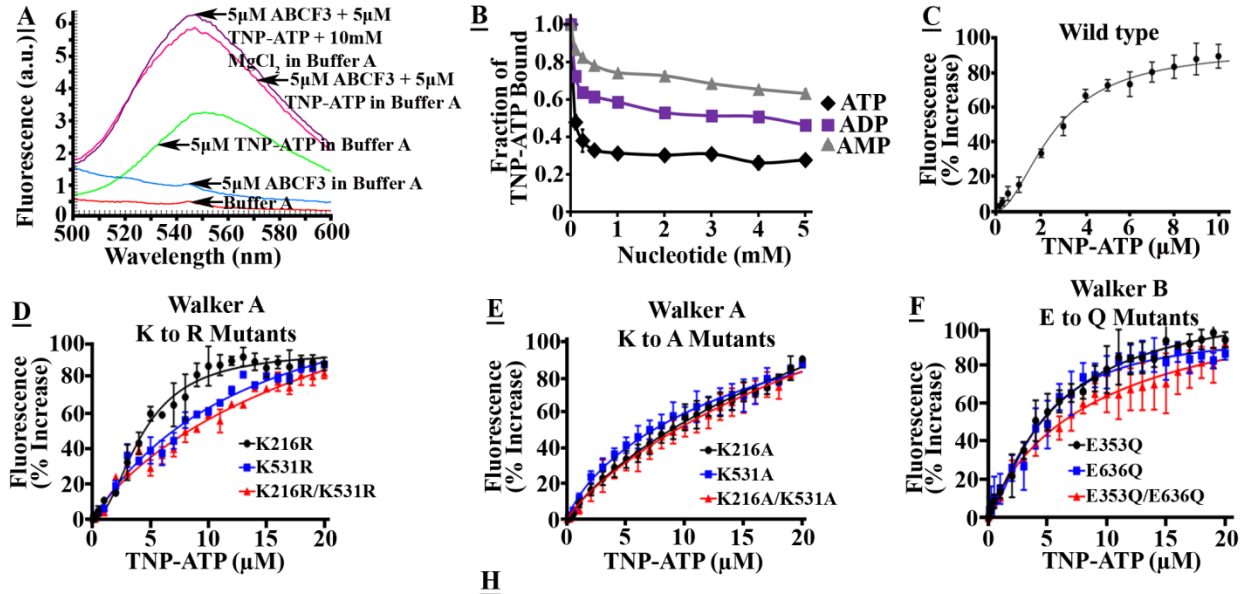
Kinetics of ATP binding to ABCF3		
Sample	K_{dATP}(µM)	Relative K_{dATP}
WT ABCF3	44 ± 1.1 µM	1.00
K216R (NBD1)	27 ± 1.0 µM	0.61
K531R (NBD2)	36 ± 1.2 µM	0.82
K216R/K531R (NBD1/NBD2)	31 ± 1.3 µM	0.70

Figure 2.5 Quenching of the intrinsic Trp fluorescence of ABCF3 by nucleotides.

A, fluorescence emission spectra for 0.5 µM ABCF3 (*purple*), 5 µM soluble Trp analog NATA (*green*) in Buffer A (1xPBS with 20% glycerol, pH 7.4), or Buffer A alone (*blue*) are shown. Excitation was carried out at 295 nm, and emission was recorded between 310 nm and 370 nm. *B*, kinetics of nucleotide binding to ABCF3 using intrinsic Trp fluorescence quenching. Increasing concentrations of ATP (*black triangle*) or ADP (*red square*) were added to a 500 µL solution of Buffer A containing 0.5 µM ABCF3. The data were corrected as described under "Experimental

procedures" and plotted using GraphPad. The data were fitted to an equation for a single binding site for both ATP and ADP. The data points represent averages of four trials. Standard deviation is within the symbols where not visible on the graph. C, *table* showing a summary of kinetics data.

To further investigate nucleotide binding, TNP-ATP, a fluorescent analog of ATP, was used. TNP-ATP alone exhibits some fluorescence in solution; however, its interaction with the nucleotide-binding pocket of a protein results in enhanced fluorescence (44,55-57). TNP-ATP binding to ABCF3 resulted in a 2-fold increase in fluorescence (in the presence or absence of 10 mM $MgCl_2$) compared with TNP-ATP in buffer (Fig. 2.6A). Moreover, a red shift in λ_{max} from 551 nm to 545 nm was also observed indicating that TNP-ATP binding occurs within a hydrophobic region in ABCF3. To determine whether TNP-ATP binds to the ATP-binding pocket(s), increasing concentrations of different nucleotides, including ATP, ADP or AMP, were added to TNP-ATP-bound ABCF3. It was expected that the addition of nucleotides would displace TNP-ATP from the binding pocket and result in a decrease in fluorescence, as reported previously (44,55-57). The addition of 0.1 mM ATP resulted in a sharp decrease in fluorescence indicating displacement of TNP-ATP (Fig. 2.6B). Significantly less displacement was seen with either 0.1 mM ADP or AMP. These results suggest that TNP-ATP binds specifically to the nucleotide-binding pocket(s) in ABCF3 and that ATP binds with higher affinity than either ADP or AMP.



H

Kinetics of TNP-ATP binding to ABCF3		
Sample	$K_{0.5}$ (μM)	nH
Wild-type ABCF3	2.6 ± 0.1 μM	1.8 ± 0.1
K216R (NBD1)	4.2 ± 0.2 μM	2.0 ± 0.2
K531R (NBD2)	13.4 ± 3.9 μM	1.0 ± 0.1
K216R/K531R (NBD1/NBD2)	28.4 ± 21.4 μM	0.9 ± 0.1
K216A (NBD1)	52.7 ± 38.0 μM	0.8 ± 0.1
K531A (NBD2)	22.1 ± 12.1 μM	0.9 ± 0.1
K216A/K531A (NBD1/NBD2)	58.6 ± 66.9 μM	0.8 ± 0.1
E353Q (NBD1)	5.7 ± 0.9 μM	1.2 ± 0.1
E636Q (NBD2)	4.2 ± 0.4 μM	1.5 ± 0.2
E353Q/E636Q (NBD1/NBD2)	9.9 ± 4.5 μM	0.9 ± 0.1
E353A (NBD1)	12.5 ± 1.4 μM	1.0 ± 0.0
E636A (NBD2)	5.9 ± 0.5 μM	1.3 ± 0.1
E353A/E636A (NBD1/NBD2)	8.9 ± 2.1 μM	1.0 ± 0.1

Figure 2.6: Binding of TNP-ATP to WT ABCF3 or mutated proteins.

A, fluorescence emission of TNP-ATP bound to ABCF3. The fluorescence emission spectrum of 5 μM TNP-ATP in Buffer A (1x PBS, 20% glycerol, pH 7.4) was examined in the presence or absence of 5 μM ABCF3. Excitation occurred at 403 nm, and emission was recorded between 450 and 600 nm. Scans carried out in the presence or absence of TNP-ATP and ABCF3 are shown. *B*, displacement of TNP-ATP by ATP, ADP, or AMP. Different concentrations of ATP (*black diamond*), ADP (*purple square*), or AMP (*gray triangle*) were added to a 500 μl solution containing 5 μM ABCF3 with 5 μM TNP-ATP and 10 mM MgCl2 after an incubation period of 5 minutes at room temperature. Fluorescence values were obtained after each addition of nucleotide

and corrected as described. The initial fluorescence (before addition of nucleotides) was set as 1.0, and the fraction of TNP-ATP bound to ABCF3 after addition of nucleotides was calculated. Each plotted value represents the average of four trials and where not visible the standard deviation lies within the data points. *C*, kinetic analysis of TNP-ATP binding to purified WT ABCF3. Titrations of ABCF3 with increasing concentrations of TNP-ATP were carried out. Aliquots of TNP-ATP were added to a 500 µl solution of 5 µM ABCF3 in Buffer A, and the fluorescence intensity (403 nm excitation, 450-600 nm emission) was recorded after each addition. Blank titrations with each TNP-ATP concentration (without addition of ABCF3) were also carried out. Fluorescence values were corrected as described under "Experimental procedures" and plotted using GraphPad Prism. *D-G*, kinetic analysis of TNP-ATP binding to mutated proteins. *D*, Walker A lysine to arginine mutations NBD1 (*black circle*); NBD2 (*blue square*); and double NBD1/NBD2 (*red triangle*). *E*, Walker A lysine to alanine. *F*, Walker B glutamic acid to glutamine. *G*, Walker B glutamic acid to alanine. Each data point represents the average of four trials. Where not visible the standard deviation lies within the data points. *H*, summary of TNP-ATP binding kinetics to WT and Walker A and B mutants fitted to allosteric sigmoidal binding model. Nonconservative mutations are shown in *red font*. The standard deviations are provided for each. *nH*=Hill Coefficient.

To determine the ABCF3 binding affinity for TNP-ATP, 5 µM ABCF3 was titrated with increasing concentrations of TNP-ATP ranging between 0.1 µM and 20 µM. TNP-ATP binding to WT ABCF3 followed sigmoidal kinetics, suggesting the presence of two nucleotide-binding sites in this protein (Fig. 2.6C). The data could be fitted to an allosteric binding model that exhibited a $K_{0.5}$ of less than 3 µM and a Hill coefficient of 1.8 (Fig. 2.6H), suggesting positive cooperativity between the two binding sites. To determine the effect of Walker A mutations on TNP-ATP

binding, titrations were also carried out with the single (NBD1 or NBD2) and double (NBD1/NBD2) mutant ABCF3 proteins. Lysine to arginine substitution mutations in the Walker A motif in each NBD of ABCF3 (K216R or K531R) resulted in higher $K_{0.5}$ values, implying a lower-binding affinity for TNP-ATP, as expected (Fig. 2.6, D and H). The K216R (NBD1) mutant protein bound TNP-ATP with a 2-fold higher $K_{0.5}$ than WT ABCF3, whereas the K531R (NBD2) mutant protein showed about a 5-fold higher $K_{0.5}$ (Fig. 2.6, D and H). The double mutant K216R/K531R protein exhibited the highest $K_{0.5}$, which was almost 10-fold higher than that of the WT protein. These data suggest that both NBDs in ABCF3 contribute to ATP binding, but NBD2 plays a more important role. Interestingly, the NBD2 mutant K531R protein provided the best fit with the single-site Michaelis-Menten model and showed a Hill coefficient of 1.0, which differed from the Hill coefficient of 1.8 observed for the WT protein. The NBD1 mutant K216R protein, however, still displayed allosteric binding of TNP-ATP with a Hill coefficient of 2.0. Together these results suggest that TNP-ATP still binds to the mutated NBD1 site (K216R) albeit with a 2-fold lower affinity, but that TNP-ATP binding to the mutated NBD2 (K531R) is significantly negatively impacted.

Since the conservative lysine to arginine mutations described above resulted in only limited loss of function of ABCF3 (especially for the NBD1 K216R mutant), nonconservative mutations of lysine to alanine were also constructed to further examine the role of each NBD. As expected, these mutations (K216A, K531A, and K216A/K531A) produced a much more drastic effect on TNP-ATP binding resulting in incomplete saturation when each protein was titrated with increasing concentrations of TNP-ATP (Fig. 2.6E). Moreover, the $K_{0.5}$ value in each case was significantly higher as compared with the lysine to arginine mutations, and the Hill coefficient in each case was <1.0 (Fig. 2.6H, shown in *red font*). The combination of the nonsaturating binding

curves seen in Fig. 2.6E and the high error in $K_{0.5}$ values reported by GraphPad in Fig. 2.6H suggested that these $K_{0.5}$ values are most likely underestimated. Overall, these results imply that TNP-ATP binding activity is severely compromised in proteins with the nonconservative lysine to alanine mutations in either NBD and confirm a role for each NBD of ABCF3 in ATP binding.

Although the Walker B motif of ABC proteins plays an important role in ATP hydrolysis (described below), the effect of single (E353Q or E636Q) and double (E353Q/E636Q) point mutations in Walker B on TNP-ATP binding was also investigated (Fig. 2.6F). As expected, the effect of conservative Walker B mutations on TNP-ATP binding was less severe than that observed for the conservative Walker A mutations. These three mutants displayed a slightly enhanced $K_{0.5}$, with the double mutant showing the largest increase (Fig. 2.6H). Nonconservative Walker B mutations (E353A, E636A, and E353A/E636A) were also examined for their effect on TNP-ATP binding. The effect of these mutations was also not as severe as that observed with the nonconservative Walker A mutations (Fig. 2.6, G and H).

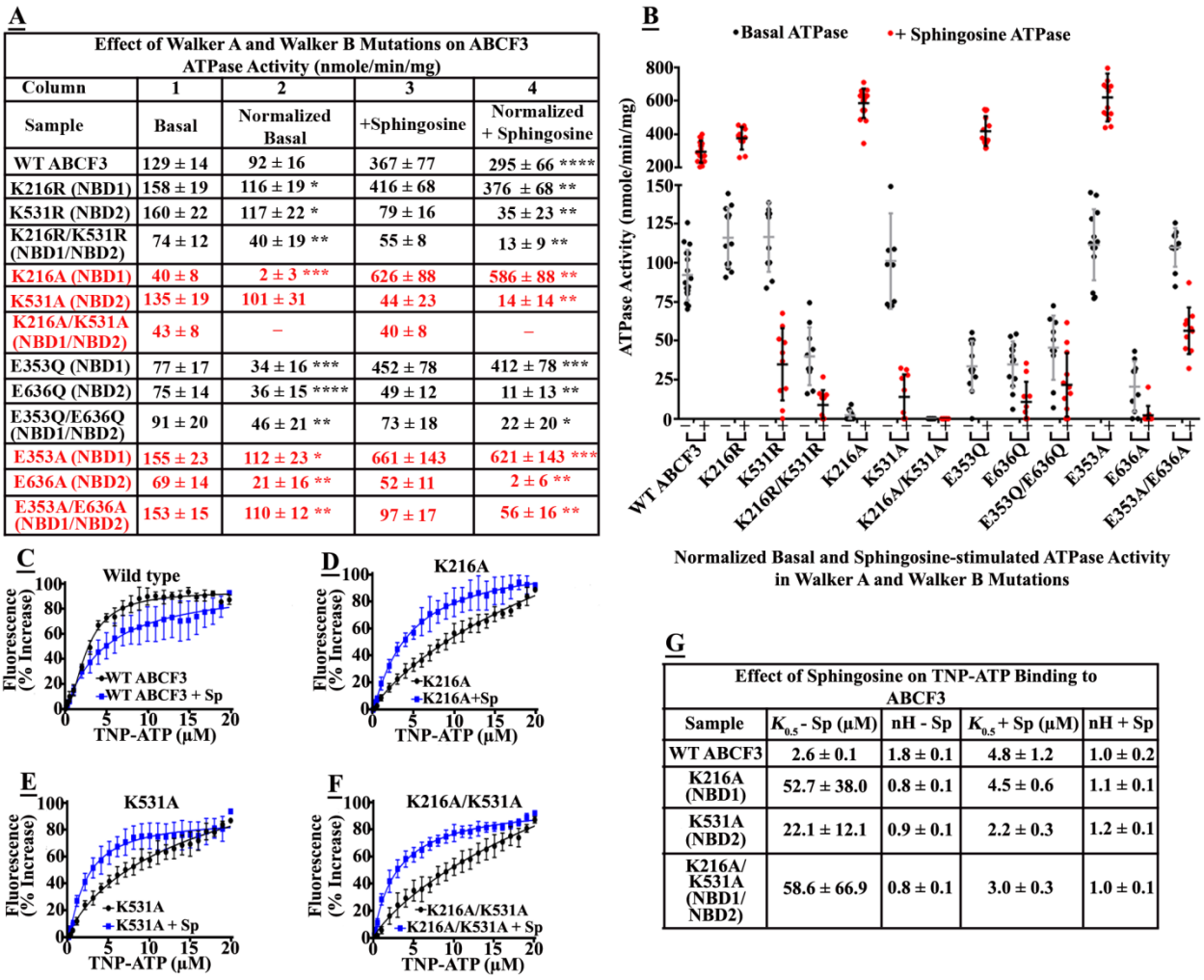
2.2.4 Role of the two NBDs of ABCF3 in ATP hydrolysis

The effect of the Walker A mutations (K216R, K531R, K216A, and K531A) or the Walker B mutations (E353Q, E636Q, E353A, and E636A) on ATPase activity was next determined. The most drastic effect on basal activity was observed for the single nonconservative point mutation, K216A, in NBD1, which resulted in less than 30% residual activity (Fig. 2.7A, *column 1*, highlighted in *red font*). In contrast, a protein with the single K531A mutation in NBD2 retained 100% of the basal activity, whereas the double mutation K216A/K531A showed less than 30% activity similar to K216A (Fig. 2.7A, *column 1*). ABCF3 proteins containing conserved double Walker A (Lys to Arg) or Walker B (Glu to Gln) mutations retained about 60-70% residual activity. The double Walker B (nonconservative Glu to Ala) mutant protein, however, showed

normal basal activity for unexplained reasons (Fig. 2.7A, *column 1*, highlighted in *red font*). Because the basal activity does not represent specific ligand-stimulated activity, the finding that the effect of various point mutations on basal activity varied is not surprising.

A

Effect of Walker A and Walker B Mutations on ABCF3 ATPase Activity (nmole/min/mg)				
Column	1	2	3	4
Sample	Basal	Normalized Basal	+Sphingosine	Normalized + Sphingosine
WT ABCF3	129 ± 14	92 ± 16	367 ± 77	295 ± 66 ****
K216R (NBD1)	158 ± 19	116 ± 19 *	416 ± 68	376 ± 68 **
K531R (NBD2)	160 ± 22	117 ± 22 *	79 ± 16	35 ± 23 **
K216R/K531R (NBD1/NBD2)	74 ± 12	40 ± 19 **	55 ± 8	13 ± 9 **
K216A (NBD1)	40 ± 8	2 ± 3 ***	626 ± 88	586 ± 88 **
K531A (NBD2)	135 ± 19	101 ± 31	44 ± 23	14 ± 14 **
K216A/K531A (NBD1/NBD2)	43 ± 8	–	40 ± 8	–
E353Q (NBD1)	77 ± 17	34 ± 16 ***	452 ± 78	412 ± 78 ***
E636Q (NBD2)	75 ± 14	36 ± 15 ****	49 ± 12	11 ± 13 **
E353Q/E636Q (NBD1/NBD2)	91 ± 20	46 ± 21 **	73 ± 18	22 ± 20 *
E353A (NBD1)	155 ± 23	112 ± 23 *	661 ± 143	621 ± 143 ***
E636A (NBD2)	69 ± 14	21 ± 16 **	52 ± 11	2 ± 6 **
E353A/E636A (NBD1/NBD2)	153 ± 15	110 ± 12 **	97 ± 17	56 ± 16 **



G

Effect of Sphingosine on TNP-ATP Binding to ABCF3				
Sample	$K_{0.5}$ - Sp (μM)	nH - Sp	$K_{0.5}$ + Sp (μM)	nH + Sp
WT ABCF3	2.6 ± 0.1	1.8 ± 0.1	4.8 ± 1.2	1.0 ± 0.2
K216A (NBD1)	52.7 ± 38.0	0.8 ± 0.1	4.5 ± 0.6	1.1 ± 0.1
K531A (NBD2)	22.1 ± 12.1	0.9 ± 0.1	2.2 ± 0.3	1.2 ± 0.1
K216A/ K531A (NBD1/ NBD2)	58.6 ± 66.9	0.8 ± 0.1	3.0 ± 0.3	1.0 ± 0.1

Figure 2.7: Effect of Walker A or Walker B mutations on ATP hydrolysis and TNP-ATP binding in the presence of 15 μM sphingosine.

A, table summarizes the basal and sphingosine-stimulated ATPase activities in nanomoles/min/mg of purified WT ABCF3 or the Walker A and Walker B mutated proteins. Same amount of purified ABCF3 protein (5 μg) was used in each assay, and the data were normalized by subtracting the

basal or sphingosine stimulated activity of K216A/K531A from WT and Walker A and Walker B mutant protein activities. The data represent the mean of at least 10 trials with the standard deviation provided. Nonconservative mutations are shown in *red font*. The results of Wilcoxon matched-pairs signed rank tests comparing normalized basal ATPase activity of WT to Walker A or B mutated proteins are shown with *asterisks* in *column 2*. The results of Wilcoxon matched-pairs signed rank tests comparing normalized basal to normalized sphingosine-stimulated ATPase activity for each protein sample are shown with *astericks* in *column 4*. **** p value ≤ 0.0001; *** p value ≤ 0.001; ** p value ≤ 0.01; * p value ≤ 0.05. *B,* scatter plot showing the normalized basal (*black circle,* -) and sphingosine-stimulated (*red circle,* +) ATPase activities in nanomoles/min/mg of Walker A and B mutant proteins. – (*black*) and + (*red*) indicate the absence or presence of sphingosine, respectively. *C-F,* kinetic analysis of TNP-ATP binding to WT ABCF3 or Walker A lysine to alanine mutated proteins with (*blue square*) or without (*black circle*) 15 μM sphingosine. *C,* WT protein. *D,* NBD1 mutant K216A protein. *E,* NBD2 mutant K531A protein. *F,* NBD1/NBD2 double mutant K216A/K531A protein. The plotted values represent the average of four trials fitted to an allosteric sigmoidal model using GraphPad Prism software. *G,* summary of TNP-ATP binding kinetics to WT and mutated proteins fitted to an allosteric sigmoidal binding model. Sp=sphingosine.

The ATPase activity of WT ABCF3 was previously shown to be stimulated by sphingosine (Fig. 2.2A), and the effect of sphingosine on the activities of the Walker A and Walker B mutant proteins was next determined. The NBD1 and NBD2 mutant proteins behaved differently after addition of sphingosine. While the ATPase activity of WT ABCF3 was stimulated about 3-fold by sphingosine, the activity of the NBD1 mutant K216A was stimulated 15-fold compared with its

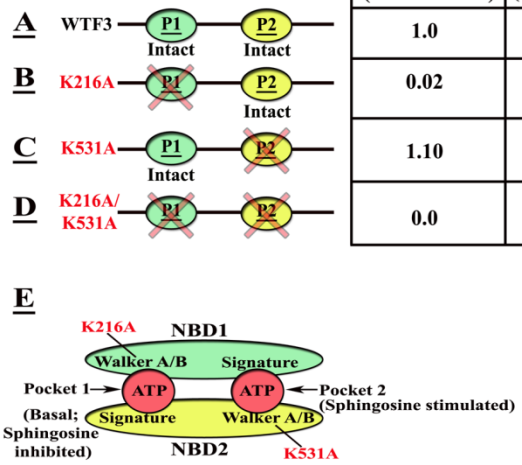
reduced basal activity (Fig. 2.7A, compare *columns 1* and *3*). The overall stimulated activity of K216A (626 nmol/min/mg) was 1.7-fold higher than the stimulated WT protein activity (367 nmol/min/mg). In contrast, the activity of the corresponding NBD2 K531A mutant protein was inhibited 3-fold by sphingosine, and the activity of the double K216A/K531A mutant protein was unaffected (Fig. 2.7A, *columns 1* and *3*). The protein with the conservative Walker A mutation K216R in NBD1 also showed about a 3-fold stimulation of activity, whereas the K531R NBD2 mutant protein showed a 2-fold decrease (Fig. 2.7A, *columns 1* and *3*). The Walker B NBD1 mutant proteins (E353A and E353Q) also showed a 4-6-fold stimulation in activity, whereas the activities of the NBD2 mutant proteins (E636A and E636Q) were decreased by about 1.5 fold, overall indicating a similar trend for the NBD1 and NBD2 mutations. Since the nonconservative double Walker A mutation K216A/K531A was most detrimental to the basal (Fig. 2.7A, *column 1*) and sphingosine-stimulated (*column 3*) ATPase activities, the residual activity of this mutant likely represents background or nonspecific activity. Therefore, the ATPase activity data were normalized by subtracting the basal and stimulated activity of the double mutant from the corresponding activities of WT ABCF3 and all other mutants (Fig. 2.7A, *columns 2* and *4*). A scatter plot of the normalized basal and sphingosine-stimulated ATPase activities of the WT and mutants is shown in Fig. 2.7B. After normalization, the ATPase activity trends remained the same. The activity of the different NBD1 mutants was stimulated by sphingosine, while the activity of different NBD2 mutants was inhibited, although the degree of fold-stimulation or inhibition was altered to varying degrees for the different mutant proteins. For example, the normalized activity of the K216A mutant protein with sphingosine was on average nearly 300-fold higher compared to its basal activity (Fig. 2.7A, *column 4*). This is due to the raw basal activities of the K216A and K216A/K531A mutants being very similar, as stated above, and thus after normalization K216A

exhibited minimal basal activity (Fig. 2.7A, *column 2*), resulting in a much higher fold-change of stimulated activity with a broader range (164-337) as shown in Fig. 2.8.

To determine whether sphingosine enhances the catalytic activity of the NBD1 mutant K216A by increasing its affinity for ATP, 5 μM of purified WT or K216A protein was titrated with increasing concentrations of TNP-ATP in the presence of 15 μM sphingosine. While the addition of sphingosine did not produce a significant change in the binding affinity of WT ABCF3 for TNP-ATP (Fig. 2.7C), the saturation curve no longer exhibited sigmoidal behavior, and the kinetics yielded a Hill coefficient of 1.0 instead of 1.8 seen in the absence of sphingosine (Fig. 2.7G). As predicted, the binding affinity of the NBD1 mutant K216A for TNP-ATP was significantly enhanced by the presence of sphingosine (Fig. 2.7, D and G). Moreover, the binding kinetics of the K216A mutant exhibited saturable binding, which is in contrast to the incomplete saturation seen in the absence of the ligand (Fig. 2.7D). The $K_{0.5}$ for K216A in the presence of sphingosine was about 10-fold lower than that seen in the absence of sphingosine and was now in the same range as for WT ABCF3. Despite the very high binding affinity, the binding curves were not sigmoidal, and the kinetic data showed a Hill coefficient of 1.1 (Fig. 2.7G). Surprisingly, addition of sphingosine also resulted in saturable binding of TNP-ATP to the NBD2 mutant K531A and the double mutant K216A/K531A with significantly reduced $K_{0.5}$ values (Fig. 2.7, E-G), even though the ATPase activities of these mutants were not stimulated by sphingosine (Fig. 2.7, A and B).

The two ATP-binding pockets in WT ABCF3 protein are shown as P1 and P2 in the linear schematic shown in Fig. 2.8A, and the negative effect of point mutations on pocket function is indicated in Fig. 2.8, B-D. The accompanying table in Fig. 2.8 summarizes the differential effects of NBD1 and NBD2 mutations on TNP-ATP binding and ATPase activities shown in Fig. 2.7.

Based on the data shown in Fig. 2.7 and Fig. 2.8, A-D, a model of the function of each pocket was generated (Fig. 2.8E). The ATP-binding pockets are expected to be located at the interface of NBD1 and NBD2 in ABCF3 and to be formed by a head-to-tail interaction previously seen in other ABC proteins (12). Pocket 1 (P1) is formed by association of Walker A and Walker B regions of NBD1 with the signature motif of NBD2, whereas pocket 2 (P2) contains the opposite arrangement. The possible implications of this model for the catalytic mechanism of ABCF3 are discussed later.



	Normalized ATPase Activity		TNP-ATP Binding			
	1	2	3	4	5	6
	Basal (relative to WT)	+ Sphingosine (relative to Basal)	$K_{0.5}$ Basal (Relative to WT)	$K_{0.5}$ + Sphingosine (Relative to WT)	nH Basal	nH +Sphingosine
A	1.0	≈ 1.81-4.14	1.0	1.0	1.8	1.0
B	0.02	≈ 164-337	≈ 20	1.0	0.8	1.1
C	1.10	≈ 0.04-0.42	≈ 9	0.5	0.9	1.2
D	0.0	0.0	≈ 23	0.6	0.8	1.0

Figure 2.8: Proposed model for the function of two ATP-binding pockets in ABCF3.

A-D, linear schematic of WT ABCF3 and proteins mutated in the Walker A motif (K216A, K531A, and K216A/K531A), each showing two ATP-binding pockets *P1* and *P2*. The mutated sites are marked with X. The ATPase activity and the TNP-ATP-binding data for WT and mutants are summarized in the *table*. The normalized activities shown in Fig. 2.7A were used to generate the relative ATPase activity values in *columns 1* and *2*. In *column 1*, the WT normalized basal ATPase values were designated as 1.0, and the relative ATPase values for each ABCF3 mutant were

calculated. In *column 2*, the fold-change was calculated by dividing the normalized sphingosine-stimulated activity of the WT and mutant ABCF3 proteins by the normalized basal activity of each protein. The range indicates variability, calculated from 10 individual experiments. Values > 1.0 indicate stimulation. Values < 1.0 indicate inhibition, with a lower value indicating greater inhibition. *Columns 3-6* are based on TNP-ATP binding analysis shown in Figure 2.7, C-G. For *column 3*, the WT $K_{0.5}$ value without sphingosine was designated as 1.0, and the relative $K_{0.5}$ values for each mutant without sphingosine are shown. In *column 4*, WT ABCF3 $K_{0.5}$ values with 15 μM sphingosine were designated as 1.0, and the relative $K_{0.5}$ values with sphingosine for each mutant are shown. *Column 5* displays the Hill coefficient (*nH*) for WT and mutant ABCF3 with TNP-ATP only, while *column 6* shows *nH* values for WT and mutant ABCF3 with TNP-ATP in the presence of 15 μM sphingosine. *E*, model of the head-to-tail interaction between the NBD1 and NBD2 domains of ABCF3 that result in the formation of two ATP-binding pockets at their interface. The locations of the NBD1 mutant K216A and NBD2 mutant K531A are indicated (*red*). Based on the data provided in the *table*, pockets 1 and 2 are proposed to be the sites for basal and sphingosine-stimulated ATPase activity, respectively. The function of pocket 1 is proposed to be inhibited by sphingosine simultaneously when pocket 2 is stimulated.

2.2.5 Co-expression of OAS1B and ABCF3 in bacteria

OAS1B protein was previously shown to be localized to the ER membrane in mammalian cells (9). This is proposed to result from the presence of a putative TM domain located at the C terminus of OAS1B (9). As bacteria contain only a cell membrane and lack organelle membranes, we hypothesized that an ABCF3-OAS1B complex formed in bacterial cells might localize to the cell membrane and provide a model for studying function, including lipid transport, by this complex. Co-expression of OAS1B and ABCF3 was analyzed in *Escherichia coli* cells. The

expression of full-length *oas1b* alone from pGEX-*oas1b* at 20°C resulted in complete growth arrest within 30 min of induction of protein expression in *E. coli* cells; however, expression of the truncated OAS1B protein lacking the putative TM domain (from pGEX-*oas1b*Δ*tm*) induced no growth inhibition (Fig. 2.9A). Western blot analysis showed significantly higher levels of the OAS1BΔTM protein compared with the full-length OAS1B (Fig. 2.9B, compare *lanes 2-4* with *lanes 5-7*). This result was expected because samples used in Fig. 2.9B, *lanes 2-4* were derived from viable cells, whereas samples in *lanes 5-7* were derived from growth-inhibited cells. Overall, the differential growth effect observed was attributed to the presence of a TMD on the full-length OAS1B protein. Most of the OAS1BΔTM protein was sequestered in the inclusion body (IB) fraction (Fig. 2.9B, *lane 2*), a phenomenon commonly seen when a heterologous protein is overexpressed in *E. coli* (58,59).

The growth inhibitory effect of full-length OAS1B was also analyzed in two other bacterial expression systems. When full-length OAS1B was expressed at 20°C from the extremely low-expression, pACYC-based, pSU2718 vector (60), growth inhibition was initially seen, but the cells recovered after about 1 hour of induction (Fig. 2.9A). Expression from pSU2718-*oas1b* at 37°C, however, resulted in no growth inhibition (Fig. 2.9A). In contrast, expression of full-length OAS1B from the higher copy number pED-*oas1b* clone at 37°C resulted in severe growth inhibition which was not reversed until 3 h (Fig. 2.9C). Growth inhibition, although to a lesser extent, was also seen when OAS1B was expressed at 30°C (Fig. 2.9D). Expression of ABCF3 alone did not have a negative effect on bacterial growth under all tested conditions (Fig. 2.9, C and D).

To determine whether co-expression of *abcf3* would impact the growth inhibitory phenotype of *oas1b* expression, the growth of *E. coli* transformed with the pED clones expressing

abcf3 (pED-*abcf3*), *oas1b* (pED-*oas1b*), or both *abcf3* and *oas1b* (pED-*oas1b-abcf3*) genes was analyzed at 37 or 30°C. Interestingly, co-expression of *oas1b* and *abcf3* completely alleviated cell growth inhibition at both 37°C (Fig. 2.9C) and 30°C (Fig. 2.9D), suggesting that an intracellular interaction between ABCF3 and OAS1B had occurred.

The cellular distribution of each expressed protein was next analyzed. The cells induced at 30 or 37°C were lysed, and the cytosolic, membrane, and IB fractions were prepared. The proteins in each fraction were separated by SDS-PAGE and detected by Western blotting with either anti-OAS1 or anti-ABCF3 antibody (Fig. 2.9, E-H). An 8-10-fold increase in the level of total (T) OAS1B in cells co-expressing *oas1b* and *abcf3*, compared with that in cells expressing *oas1b* alone, was observed both at 30 and 37°C (Fig. 2.9, G and H, compare *lanes 5* and *9*, and K and L, *T*). However, the majority of the OAS1B protein produced under co-expression conditions was found in the IB fraction with about a 25-fold increase in the accumulation of OAS1B in IB at 37°C compared to expression of OAS1B alone (Fig. 2.9, G, compare *lanes 2* and *6*, and K, *IB*). At 30°C, a 20-fold higher level of OAS1B was observed in the IB fraction (Fig. 2.9, H, compare *lanes 2* and *6*, and L, *IB*) with lower OAS1B levels detected in the cytosolic and membrane fractions. The results indicate that due to the sequestration of over-expressed OAS1B in inclusion bodies at both temperatures, OAS1B localization to the membrane remained the same or decreased in co-expressing cells. Moreover, co-expression at 30 or 37°C had little or no effect on the stability or cellular distribution of ABCF3 (Fig. 2.9, I and J).

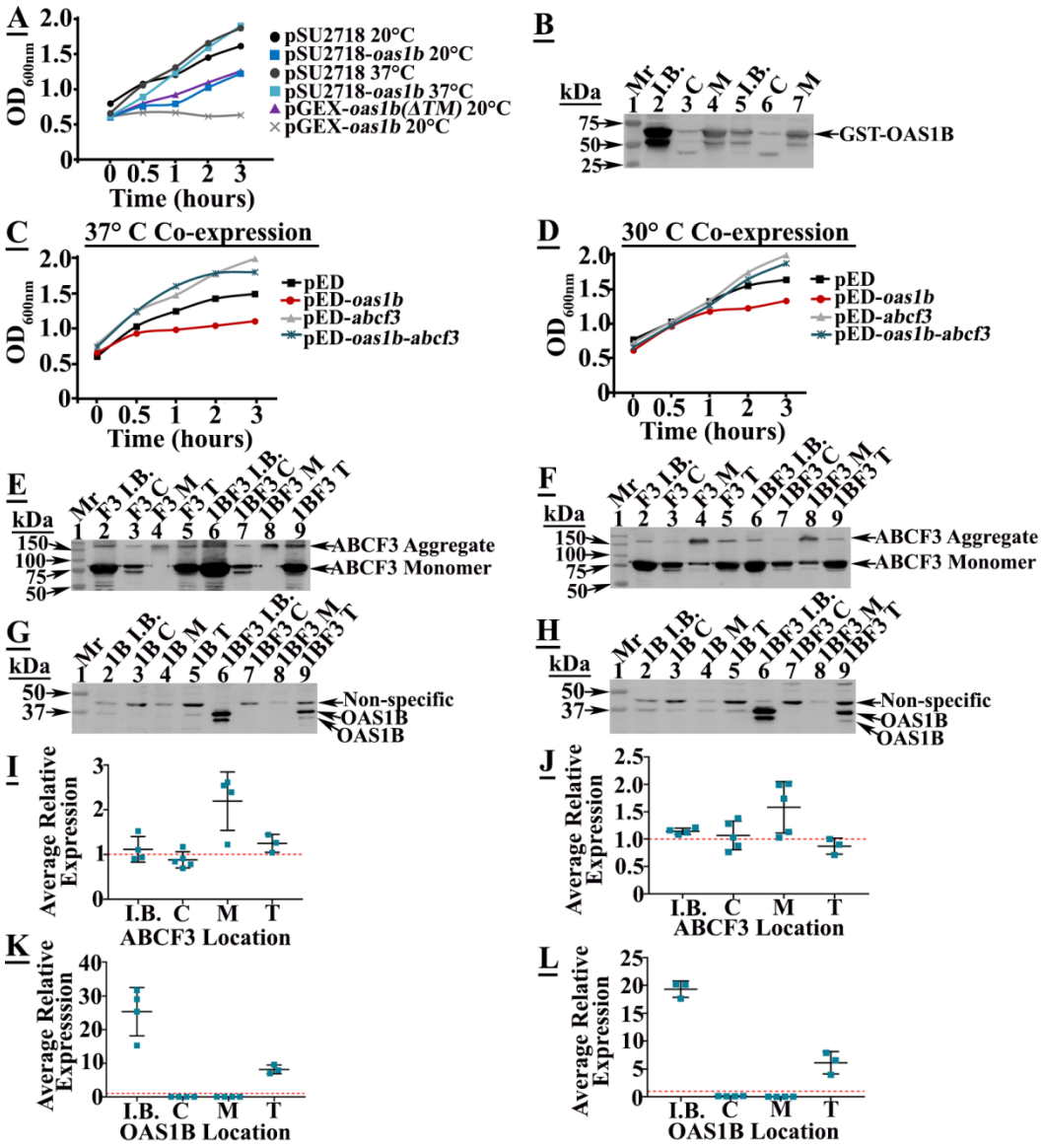


Figure 2.9: Co-expression of oas1b and abcf3 in bacteria.

A and B, effect of expression of full-length OAS1B on growth of *E. coli* cells. *A*, Rosetta 2(DE3)pLysS cells containing pGEX-*oas1b* (*gray times sign*) or pGEX-*oas1b(Δtm*) (*purple triangle*) or BL21 cells containing pSU2718 (*black circle*) or pSU2718-*oas1b* (*blue square*) were grown at 37 °C to mid-log phase ($A_{600\ nm}$ = 0.6) and induced with 0.25 mM IPTG for 3 h at 20 °C. BL21 cells containing pSU2718 (*dark gray circle*) or pSU2718-*oas1b* (*cyan square*) were also

separately induced with 0.25 mM IPTG for 3 h at 37°C. Cell growth was monitored for 3 h after induction. A representative growth experiment is shown. *B,* Western blot analysis using anti-OAS1 antibodies. pGEX-*oas1b(Δtm*) or pGEX-*oas1b*-containing cells collected at the 3 h time point in *A* were fractionated as described to obtain the inclusion body (*I.B.*), cytosol (*C*), and membrane (*M*) fractions. Twenty five μg of each sample was loaded on 10% SDS-polyacrylamide gels, followed by Western blotting against anti-OAS1 (1:500) antibodies as described under "Experimental procedures." *Lane 1*, marker (Mr); *lanes 2-4*, OAS1B(ΔTM); *lanes 5-7*, OAS1B. *C and D,* effect of co-expression of OAS1B and ABCF3 on growth. *E. coli* Rosetta 2(DE3)pLysS cells containing pED (*black square*), pED-*oas1b* (*red circle*), pED-*abcf3* (*gray triangle*), or pED-*oas1b-abcf3* (*teal times sign*) were grown at 37 °C to mid-log phase ($A_{600\ nm}$ = 0.6) and induced with 0.25 mM IPTG for 3 h at 37 °C *(C)* or 30°C *(D)*. The growth was monitored at $A_{600\ nm}$ for 3 h after induction, and a representative growth experiment is shown. *E-H,* Western blot analysis of the levels of ABCF3 and OAS1B in cell fractions at 37 °C (*left panels*) or 30 °C (*right panels*). Samples collected at the 3-h time point in *C* and *D* were fractionated to obtain the inclusion body (*I.B.*), cytosol (*C*), and membrane (*M*) fractions. *T,* total sample. Samples were loaded on 10% SDS-polyacrylamide gels, followed by Western blotting with anti-ABCF3 (1:2000) or anti-OAS1 (1:500) antibodies. *E and F,* Western blot analysis using anti-ABCF3. Fifty μg proteins were loaded with the exception of the *I.B.* samples where 25 μg proteins were loaded. *Lane 1*, marker (Mr); *lanes 2-5*, ABCF3; *lanes 6-9*, OAS1B/ABCF3. *G and H,* Western blot analysis using anti-OAS1. Fifty μg proteins were loaded in each lane, as described above for *E* and *F*. *Lane 1*, marker (Mr); *lanes 2-5*, OAS1B; *lanes 6-9*, OAS1B/ABCF3. *I-L,* scatter plots showing average relative expression of ABCF3 (*I* and *J*) and OAS1B (*K* and *L*) at 37 °C (*left panels*) or 30 °C (*right panels*). For comparison of protein levels in single and co-expression experiments, samples from all trials

were electrophoresed together on the same gel and analyzed by densitometric scanning using Multi-Guage version 2.3 software to obtain intensity values. The protein level obtained under the single expression condition in each case was designated as 1.0, which is shown as a *dotted red line* in the scatter plots for comparison with co-expression samples. Average intensity values obtained from $\geq$ 3 trials were then plotted. *1B*=OAS1B; *F3*=ABCF3; *1BF3*=OAS1B/ABCF3. The clones used in this figure were created by Chao Zhao.

To promote localization of the OAS1B protein to the bacterial membranes, two TMDs of the *E. coli* FtsH protein were fused to the OAS1BΔTM protein to generate the OAS1BΔTM-2TMFtsH protein in the pED-*oas1bΔtm* vector. After the fusion clone pED-*oas1bΔtm-2tmftsh* was produced it was then subcloned into pED-*abcf3* for coexpression with ABCF3. These plasmids were used to compare the effect on growth and protein localization resulting from expression of the wild type OAS1B or fusion protein alone with or without expression of ABCF3 in bacterial cells. The growth of *E. coli* cells transformed with the pED clones expressing *abcf3*, *oas1bΔtm-2tmftsh*, or co-expressing both *abcf3* and *oas1bΔtm-2tmftsh* were analyzed at 37°C or 30°C (Fig. 2.10A and Fig. 2.11A). Similarly, to *oas1b* expression, *oas1bΔtm-2tmftsh* expression inhibited cell growth at both temperatures. However, at 30°C the growth inhibition recovered after 2 hours of induction. Interestingly, co-expression of *oas1bΔtm-2tmftsh* and *abcf3* completely alleviated cell growth inhibition at both 37°C (Fig. 2.10A) and 30°C (Fig. 2.11A), as observed previously when *oas1b* and *abcf3* are co-expressed (Fig. 2.9, C and D).

The cellular distribution of each expressed protein was also analyzed for cells induced at 30 or 37°C. These cells were lysed to isolate the cytosolic, membrane, and IB fractions, which were separated by SDS-PAGE, followed by detection by Western blotting with either anti-OAS1

or anti-ABCF3 antibody (Fig. 2.10 and 2.11, B-I). As observed with OAS1B, the majority of the OAS1BΔTM-2TMFtsH protein produced under co-expression conditions was found in the IB fraction at both 37°C and 30°C growth conditions. Furthermore, co-expression had little or no effect on the cellular distribution of ABCF3 (Fig. 2.10 and 2.11, B-I).

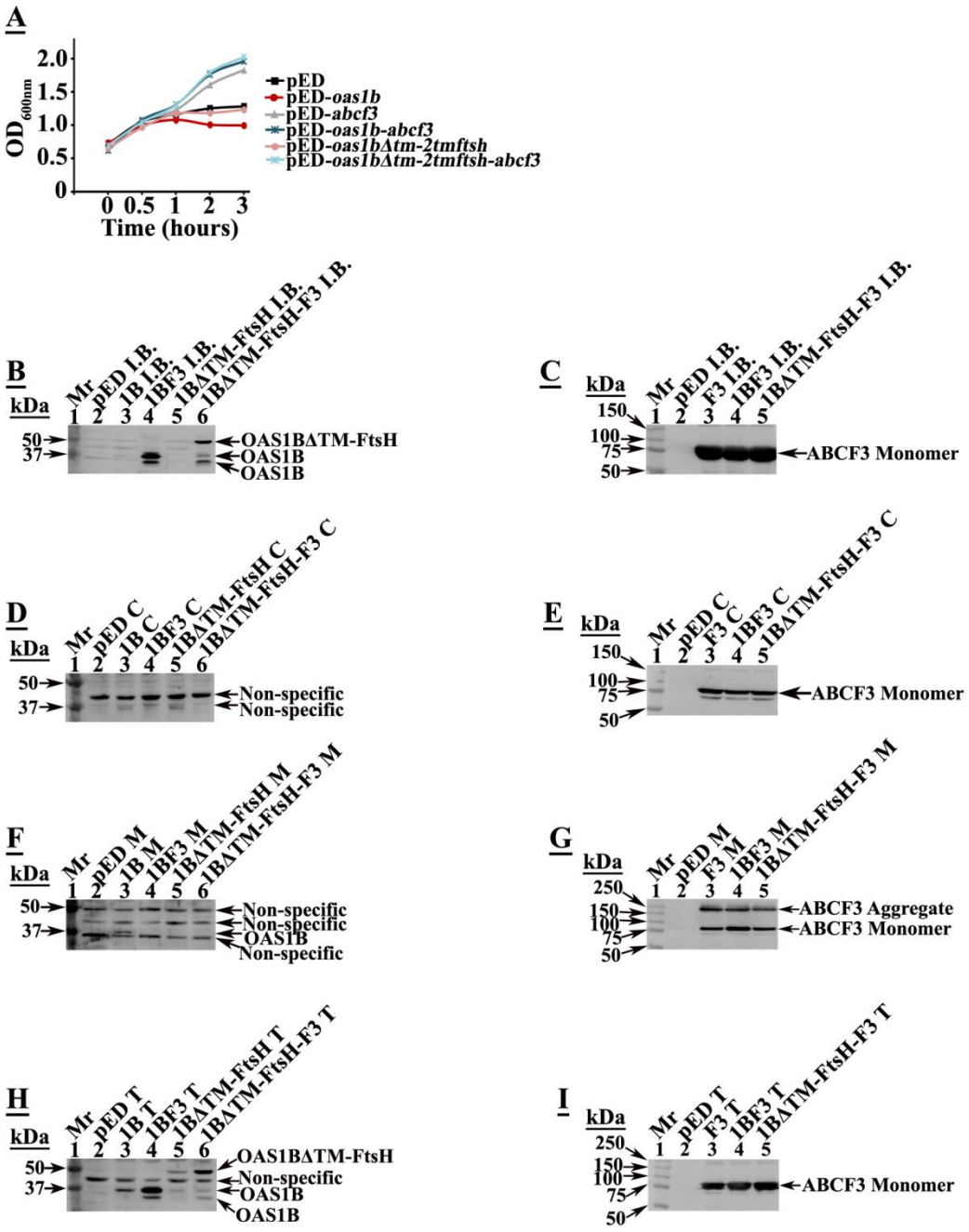


Figure 2.10: Co-expression of oas1bΔtm-2tmftsh and abcf3 in bacteria at 37°C.

Co-expression of *oas1bΔtm-2tmftsh* and *abcf3* in bacteria at 37°C. The two transmembrane domains of the *E. coli* FtsH protein were fused to the truncated OAS1BΔTM protein. A, Effect of co-expression of OAS1BΔTM-2TMFtsH and ABCF3 on growth. *E. coli* Rosetta 2(DE3)pLysS

cells containing pED (*black square*), pED-*oas1b* (*red circle*), pED-*abcf3* (*gray triangle*), pED-*oas1b-abcf3* (*teal times sign*), pED-*oas1bΔtm-2tmftsh* (*pink circle*), or pED-*oas1bΔtm-2tmftsh-abcf3* (*light blue times sign*) were grown at 37 °C to mid-log phase ($A_{600\ nm}$ = 0.6) and induced with 0.25 mM IPTG for 3 h at 37 °C. The growth was monitored at $A_{600\ nm}$ for 3 h after induction, and a representative growth experiment is shown. B, D, F, H, Western blot analysis of the levels of OAS1B and OAS1BΔTM-2TMFtsH in cell fractions at 37 °C. Samples collected at the 3 h time point in *A* were fractionated to obtain the inclusion body (*I.B.*) in *B*, cytosol (*C*) in *D*, and membrane (*M*) fractions in *F*. *T*, total sample shown in *H*. Samples were loaded on 10% SDS-PAGE gels, followed by Western blotting with anti-OAS1 (1:500) antibodies. Fifty μg proteins were loaded in each lane. *Lane 1*, marker (Mr); *lane 2*, pED only, *lane 3*, OAS1B, *lane 4*, OAS1B/ABCF3; *lane 5*, OAS1BΔTM-2TMFtsH, *lane 6*, OAS1BΔTM-2TMFtsH/ABCF3. C, E, G, I, Western blot analysis using anti-ABCF3 (1:2000). Fifty μg proteins were loaded with the exception of the *I.B.* samples where 25 μg proteins were loaded. *Lane 1*, marker (Mr); *lane 2*, pED only; *lane 3*, ABCF3; *lane 4*, OAS1B/ABCF3; *lane 5*, OAS1BΔTM-2TMFtsH/ABCF3. The pED-*oas1bΔtm-2tmftsh* clone and the pED-*oas1bΔtm-2tmftsh-abcf3* clones were created by Elizabeth Peterson, while the other clones used in this figure were created by Chao Zhao.

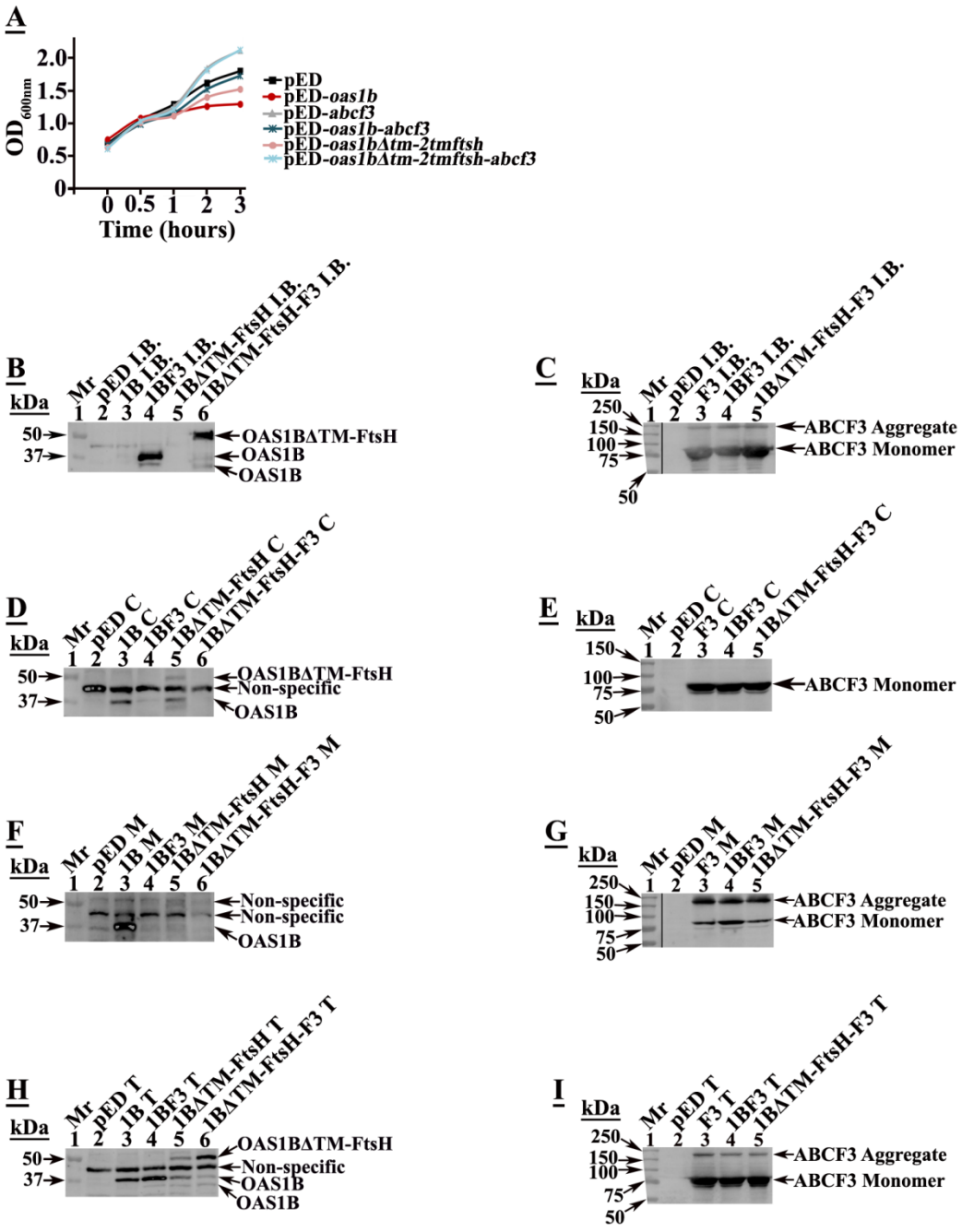
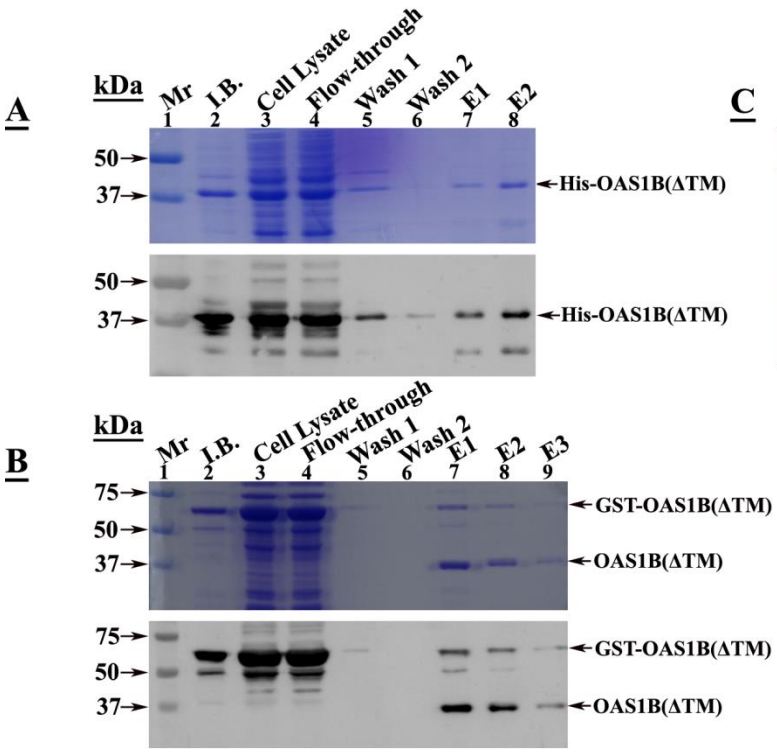


Figure 2.11: Co-expression of oas1bΔtm-2tmftsh and abcf3 in bacteria at 30°C.

Co-expression of *oas1bΔtm-2tmftsh* and *abcf3* in bacteria at 30°C. The two transmembrane domains of the *E. coli* FtsH protein were fused to the truncated OAS1BΔTM protein. A, Effect of co-expression of OAS1BΔTM-2TMFtsH and ABCF3 on growth. *E. coli* Rosetta 2(DE3)pLysS cells containing pED (*black square*), pED-*oas1b* (*red circle*), pED-*abcf3* (*gray triangle*), or pED-

oas1b-abcf3 (*teal times sign*), pED-*oas1bΔtm-2tmftsh* (*pink circle*), and pED-*oas1bΔtm-2tmftsh-abcf3* (light blue times sign) were grown at 37 °C to mid-log phase ($A_{600\ nm}$ = 0.6) and induced with 0.25 mM IPTG for 3 h at 30 °C. The growth was monitored at $A_{600\ nm}$ for 3 h after induction, and a representative growth experiment is shown. B, D, F, H, Western blot analysis of the levels of OAS1B and OAS1BΔTM-2TMFtsH in cell fractions at 30 °C. Samples collected at the 3 h time point in *A* were fractionated to obtain the inclusion body (*I.B.*) in *B*, cytosol (*C*) in *D*, and membrane (*M*) fractions in *F*. *T*, total sample shown in *H*. Samples were loaded on 10% SDS-PAGE gels, followed by Western blotting with anti-OAS1 (1:500) antibodies. Fifty μg proteins were loaded in each lane. *Lane 1*, marker (Mr); *lane 2*, pED only, *lane 3*, OAS1B, *lane 4*, OAS1B/ABCF3; *lane 5*, OAS1BΔTM-2TMFtsH, *lane 6*, OAS1BΔTM-2TMFtsH/ABCF3. C, E, G, I, Western blot analysis using anti-ABCF3 (1:2000). Fifty μg proteins were loaded with the exception of the *I.B.* samples where 25 μg proteins were loaded. *Lane 1*, marker (Mr); *lane 2*, pED only; *lane 3*, ABCF3; *lane 4*, OAS1B/ABCF3; *lane 5*, OAS1BΔTM-2TMFtsH/ABCF3. The pED-*oas1bΔtm-2tmftsh* clone and the pED-*oas1bΔtm-2tmftsh-abcf3* clones were created by Elizabeth Peterson, while the other clones used in this figure were created by Chao Zhao.



C

ATPase Activity of Purified OAS1B(ΔTM)		
Protein	Yield (mg/ml)	ATPase Activity (nmole/min/mg)
OAS1B(ΔTM) (GST Tag Removed)	0.8	87 ± 5
His-OAS1B(ΔTM)	0.5	91 ± 5

Figure 2.12: Expression and purification of OAS1B.

Protein purification and ATPase activity assays were performed as described under "Experimental procedures." A, *E. coli* HMS174(DE3) cells containing pET28a-*oas1b(Δtm)* were used for purification of OAS1B. Samples 2-6 were normalized by volume. Equal volumes (10 µl) were then analyzed using 10% SDS-PAGE, followed by staining with Coomassie Blue and Western blot analysis using anti-OAS1 (1:500). Two µl of the 1 ml fractions were analyzed in *lanes 7-8*. *Lane 1*, Mr (marker); *lane 2* (I.B.) inclusion body; *lane 3*, cell lysate; *lane 4*, column flow-through; *lane 5*, washed with 50 ml of 30 mM imidazole in Buffer A (1x PBS, 20% glycerol, pH 7.4); *lane 6*, washed with 1 ml of 100 mM imidazole in Buffer A; *lanes 7-8*, 1 ml of the elution fractions, each with 500 mM imidazole in Buffer A. *B, E. coli* Rosetta 2(DE3)pLysS cells containing pGEX-*oas1b(Δtm)* were used for purification of OAS1B with GST-tag cleavage during elution. Samples 2-6 were normalized by volume. Equal volumes (10 µl) were then analyzed using 10% SDS-PAGE, followed by staining with Coomassie Blue and Western blot analysis using anti-OAS1

(1:500). Two µl of 1 ml elution was analyzed in *lanes 8-10*. *Lane 1*, Mr; *lane 2*, (I.B.) inclusion body; *lane 3*, cell lysate; *lane 4*, column flow-through; *lane 5*, washed with 30 ml of Buffer A; *lane 6*, washed with 20 ml Buffer B (1x cleavage buffer, 20% glycerol, pH 7.0); *lanes 7-9*, elution fractions 1-3 with 1 ml of Buffer B after GST tag cleavage with PreScission Protease (GE Healthcare). *C, table* showing the yield of purified OAS1B and mean ± S.D. n=10 experiments for 5 µg basal OAS1B ATPase activity. A Mann-Whitney test showed there was no significant difference between OAS1B(ΔTM) and His-OAS1B(ΔTM) basal ATPase activity, *p* value 0.1256. The pGEX-*oas1bΔtm* clone was created by Chao Zhao, and the pET28a-*oas1bΔtm* was created by Emma Shippee.

2.3 Discussion

Recent studies have demonstrated the involvement of the full-length OAS1B protein in conferring a flavivirus resistance phenotype in mice (9,61,62). In yeast two-hybrid and subsequent *in vitro* pulldown experiments, ABCF3 and ORP1L were identified as potential OAS1B partners that may play a role in the flavivirus resistance mechanism (9). However, the specific roles of these partners in the resistance phenotype have not been determined.

In this study, a nonhydrolyzable analog TNP-ATP was used to gain an understanding of the nucleotide-binding properties of ABCF3. Interestingly, we found that TNP-ATP binding to ABCF3 follows allosteric kinetics and exhibits positive cooperativity with a Hill Coefficient of 1.8. The two NBDs in ABCF3 are each thought to participate in forming an ATP-binding pocket (Fig. 2.8E), and the data obtained suggest cooperativity between the two pockets with binding of a nucleotide to one pocket in ABCF3 increasing the binding affinity of the other pocket. Conservative mutations (Lys to Arg) in the Walker A motif of either NBD resulted in a decrease

in affinity for TNP-ATP, implying a role for each NBD in nucleotide binding. However, the NBD2 mutation (K531R) produced a much larger effect on TNP-ATP binding, suggesting an unequal contribution of the two NBDs. Results showing an unequal contribution of the two NBDs to the function of the bacterial ABCF protein Vga(A) were previously reported (38). Moreover, the NBD2 mutation in Vga(A) was found to be more detrimental than the NBD1 mutation, as seen in the case of ABCF3.

We determined that ABCF3 is an active ATPase with a basal ATPase activity of about 130 nmol/min/mg. Modulation of ABCF3 activity by several lipids and alkyl ether lipid-based amphiphilic drugs was observed, suggesting an ability of ABCF3 to directly bind these lipids and drugs. Although sphingosine, sphingomyelin, PAF, and LPC enhanced the activity, the alkyl ether lipids miltefosine, edelfosine, and perifosine, as well as LPI, lyso PAF, and cholesterol either inhibited activity or produced a biphasic response. Alkyl ether lipids are derived from the glycerophospholipid LPC (63) and the results suggest that small changes in lipid structure can produce different effects on ABCF3 activity. Although it is currently not understood why some lipids enhance while others inhibit ABCF3 ATPase activity, differential effects of different substrates on the activities of other ABC proteins have been observed (45,64,65). Strong inhibition of the ATPase activity of Vga(A) and other ABCF proteins by their antibiotic substrates reported previously also suggested direct interaction with their substrates (22,38).

Point mutations in the NBD1 and NBD2 of ABCF3 affected both basal and ligand-stimulated ATPase activity differently providing further evidence for the asymmetric nature of the two NBDs. Specifically, the NBD1 mutant K216A protein (containing intact pocket 2, Fig. 2.8B) exhibited significantly reduced basal activity, while the NBD2 mutant K531A protein (containing intact pocket 1, Fig. 2.8C) showed full basal activity. Furthermore, addition of sphingosine to

proteins containing mutations in NBD1 resulted in a significantly higher stimulation of activity than observed with the WT protein (Fig. 2.8B), while the activity of proteins containing NBD2 mutations was not only unstimulated but was inhibited in response to sphingosine (Fig. 2.8C). Sphingosine also did not stimulate the ATPase activity of the K216A/K531A double mutant (Fig. 2.8D). Based on these observations, we assume that the 100% basal ATPase activity seen in K531A mutation results from the intact pocket P1 (Fig. 2.8C), and the high sphingosine stimulation seen in K216A comes from the intact pocket P2 (Fig. 2.8B). Therefore, we propose that pocket 1 is the site of basal catalysis, whereas pocket 2 engages in ligand-stimulated ATP hydrolysis (Fig. 2.8E). The inhibition of the ATPase activities of the NBD2 mutant proteins also suggests that sphingosine binding produces a dual effect, stimulating the ATPase activity of pocket 2 while inhibiting the activity of pocket 1 (Fig. 2.8, C and E).

The above data are consistent with the TNP-ATP binding analysis conducted in the presence of sphingosine. While sphingosine restored the binding affinity of the K216A mutant protein to WT levels, the binding occurred with a Hill coefficient of 1.1, suggesting that the enhanced TNP-ATP binding in the presence of sphingosine occurs predominantly to the intact pocket 2 (Fig. 2.8B). Interestingly, sphingosine-dependent TNP-ATP binding to WT ABCF3 also demonstrated a Hill coefficient of 1.0 in contrast to the cooperative binding seen in the absence of the ligand (Fig. 2.8A), indicating that in the presence of sphingosine only one site preferentially binds ATP, and this active site corresponds to pocket 2. Surprisingly, addition of sphingosine to the pocket 2 mutant (K531A) or the double mutant (K216A/K531A) protein also resulted in overall high affinity TNP-ATP binding. In contrast to the K216A mutant protein, however, neither the pocket 2 mutant nor the double mutant showed any stimulation of ATPase activity by sphingosine (Fig. 2.8, C and D). Because TNP-ATP binding normally occurs with a much higher affinity than

ATP binding (44,55,56), the simplest explanation for these data may be that sphingosine can enhance TNP-ATP binding to pocket 2 despite the presence of the K531A mutation, but it is unable to restore its catalytic function. Overall, these data indicate the importance of an intact pocket 2 in sphingosine-stimulated ATP binding and catalysis by ABCF3.

Both co-immunoprecipitation of OAS1B and ABCF3 from mammalian cells and co-localization of OAS1B and ABCF3 at the ER membrane of mammalian cells were previously shown (9). The OAS1B-tr protein, which does not contain a C-terminal transmembrane domain, is unable to localize to the ER and does not confer flavivirus resistance. Knockdown of ABCF3 in infected cells resulted in an increase in WNV yields but did not affect the yields of two nonflaviviruses, indicating that the action of ABCF3 is specific for flaviviruses. Furthermore, the effect of ABCF3 knockdown on WNV yields was observed in WNV-infected MEFs expressing full-length OAS1B but not in infected MEFs expressing OAS1B-tr, suggesting that interaction between ABCF3 and OAS1B at the ER plays a role in the OAS1B-mediated flavivirus resistance phenotype in infected cells (9). The data presented here provide strong, though indirect, evidence for interaction between OAS1B and ABCF3 in bacterial cells. We showed that the expression of OAS1B alone in *E. coli* results in varying degrees of growth inhibition, including complete growth arrest, depending on the copy number of the vector and the temperature of expression. This phenotype is consistent with the inhibitory effect produced by overexpression of some membrane proteins in bacterial cells (58,66). Removal of the C-terminal domain of OAS1B containing the putative TM domain resulted in alleviation of growth inhibition, providing support for the proposal that OAS1B is a membrane-embedded protein (9). Furthermore, co-expression of full-length OAS1B with ABCF3 rescued the growth inhibitory phenotype produced by OAS1B expression alone at 30°C or 37°C, suggesting interaction between OAS1B and ABCF3. Co-expression also

unexpectedly resulted in a striking increase in the cellular levels of OAS1B, indicating that ABCF3 protects OAS1B from degradation by cellular proteases. The majority of the OAS1B protein stabilized under the co-expression growth conditions at either 30 or 37°C was, however, sequestered in an insoluble fraction in the cell. It is well-documented that the expression or overexpression of a heterologous membrane protein in bacteria can often result in toxic effects (67,68), proteolysis by housekeeping proteases (69-72), and/or accumulation of the overexpressed protein in inclusion bodies (58,59). Co-expression with an interacting partner protein has been previously shown to result in alleviation of toxicity and protection from proteolysis (69,72,73). We saw evidence of all these phenomena under different expression conditions: toxicity and proteolysis of OAS1B when it was expressed alone but alleviation of growth inhibition and stabilization of OAS1B, followed by sequestration in inclusion bodies, when co-expressed with ABCF3.

To our knowledge, interaction between eukaryotic proteins in bacterial cells has not been shown previously. The pETDuet-1-based bacterial co-expression system described here is not only ideal for examining protein complexes (74-76), but it also offers several distinct advantages for advancing knowledge of the two proteins. The availability of a clear growth phenotype (growth inhibition/rescue) could be used to develop a genetic screen for further analyzing the domains involved in interaction between OAS1B and ABCF3. For example, the linker domain of ABCF3 may play a role in interaction with OAS1B. The effect of mutations and/or deletions in this and other domains of either ABCF3 or OAS1B could be tested in the bacterial system by a simple growth inhibition/rescue assay. Furthermore, stabilization of large amounts of OAS1B by ABCF3 and the resulting sequestration in inclusion bodies was unexpected, and this could be utilized to prepare large amounts of OAS1B for biochemical and structural analysis in the future. Some

eukaryotic proteins have previously been genetically manipulated to promote inclusion body formation and then recovered from inclusion bodies by solubilization and refolding into a functional form (77,78). Functional integration of OAS1B and ABCF3 into bacterial membranes may also be achievable in the future through further optimization of low-level expression (66,79,80) as was previously shown for G protein-coupled receptors (81-83).

In conclusion, we showed that the mouse ABCF3 is an active ATPase, and its activity is modulated by several lipids, including sphingosine and sphingomyelin, two lipids previously shown to have altered levels in flavivirus infected cells (50-52). High levels of ATP have been shown to be required for efficient viral RNA synthesis inside membrane replication vesicles (84,85). The dengue NS3 helicase unwinds dsRNA templates in the presence of high levels of ATP but anneals complementary RNA strands when ATP levels are low (86). Although OAS1B protein is not an active 2-5A synthetase, we found it to have an ATPase activity of about 90 nmol/min/mg (Fig. 2.12, A-C). Therefore, the ABCF3-OAS1B complex, which is anchored in the endoplasmic reticular membrane, may contribute to the reduced level of viral RNA production characteristic of the flavivirus resistance phenotype through its ATP binding and hydrolysis activities, which may be modulated by lipids as shown in this study.

2.4 Experimental procedures

2.4.1 Reagents and Antibodies

All reagents were purchased from Sigma-Aldrich unless otherwise noted. 2′(3′)-*O*-(2,4,6-Trinitrophenyl)adenosine 5′-triphosphate (TNP-ATP) was purchased from Molecular Probes, Inc. and stored at -20°C in the dark; Hoechst 33342 was from Invitrogen; edelfosine, cholesterol, 10:0 phosphatidylcholine (10:0 PC), 14:0 phosphatidylethanolamine (14:0 PE), LPC, LPI, sphingosine, sphingosine-1-phosphate, sphingomyelin, PAF, lyso PAF, ceramide and dihydroceramide were

from Avanti Polar Lipids, Inc.; Glutathione Sepharose 4B and PreScission Protease were from GE Healthcare; and HisPur^{TM} Ni-NTA resin was purchased from Thermo Scientific. Nucleotides (pH 7.5) and drugs were prepared in distilled deionized water unless otherwise stated. Cholesterol, sphingosine, sphingosine-1-phosphate, PAF, lyso PAF, LPC, LPI, alkyl ether lipids, ceramide, dihydroceramide, and quinidine were prepared in ethanol prior to use. 10:0 PC and 14:0 PE were prepared in a buffer consisting of 50 mM MOPS, 125 mM NaCl, pH 7.5 and sonicated before use.

2.4.2 Subcloning of abcf3 and oas1b

A TOPO® XL PCR Cloning Kit (Invitrogen) was used to clone *abcf3* or *oas1b* into the pCR®-XL-TOPO® vector (pCR). The *abcf3* gene was subcloned from pCR-*abcf3* into pUC18 using EcoRI and XbaI, into pET-Duet-1 (pED) using NdeI and AvrII, and into pGEX-6p-1(pGEX) using EcoRI and XhoI restriction sites. *abcf3* was then subcloned from pGEX-*abcf3* into pET28a using EcoRI and XhoI restriction sites. The pGEX-*abcf3* and pET28a-*abcf3* clones express ABCF3 containing an N-terminal GST-tag and His-tag, respectively.

The *oas1b* gene was subcloned from pCR-*oas1b* into pSU2718 using PstI and HindIII, into pED using NcoI and BamHI, and into the pGEX-6p-1 vector using BamHI and EcoRI restriction sites. A C-terminally truncated version of *oas1b*, named *oas1b(Δtm),* was amplified using a forward primer containing a BamHI restriction site and a reverse primer containing a stop codon after nucleotide 1059 of *oas1b* followed by an EcoRI restriction site. The *oas1b(Δtm)* fragment was then subcloned into the pGEX-6p-1 vector using BamHI and EcoRI restriction sites to generate pCR-*oas1b(Δtm)*. To create the double expression clone pED-*oas1b-abcf3*, *oas1b* from pCR-*oas1b* was subcloned into pED-*abcf3* using NcoI and BamHI restriction sites. The pED clones (referred to as pED*1b*, pED*f3*, and pED*1bf3*) express ABCF3 and/or OAS1B protein without a tag.

2.4.3 Media, growth, isolation, and analysis of cell fractions

E.coli Rosetta 2(DE3)pLysS cells containing pED, pED*1b*, pED*f3*, or pED*1bf3* were grown in 50 ml LB medium with ampicillin (100 μg/ml) at 37°C overnight. The next day these cultures were diluted 1:50 into 250 ml of fresh LB with ampicillin in a 1 L flask and incubated at 37°C until the mid-log phase was reached ($A_{600\ nm}$ =0.6). The cultures were then induced with 0.25 mM IPTG and incubated at 37°C or 30°C for three hours following induction. Cells in 100 ml of culture media obtained under different growth conditions were pelleted by centrifugation. The pellets were resuspended in 3 ml of 1x PBS buffer, pH 7.4 containing 20% glycerol (Buffer A), 1 mM DTT, and protease cocktail inhibitor (Roche Diagnostics). Samples were lysed twice by passage through a mini French press cell (Thermo Electron Corporation) at 16,000 psi to obtain a total cell lysate. After centrifugation at 13,000 x g for 20 minutes at 4°C, the inclusion body (pellet, IB) was collected and the supernatant was centrifuged at 100,000 x g for 1 hour to obtain the cytosol (supernatant) and the membrane (pellet). The membrane and the inclusion body pellets were resuspended in 250 μl and 500 μl, respectively, of Buffer A containing 1 mM DTT. The protein concentration of each fraction was determined with a DC™ assay (Bio-Rad).

2.4.4 Western Blot analysis

ABCF3 or OAS1B in cellular fractions and as purified proteins were detected by Western blotting. Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to a nitrocellulose membrane for 16 hours at 4°C. An equal amount of protein was loaded per well unless otherwise indicated in the figure legends. After transfer, the membranes were blocked with 0.2% non-fat dry milk for ABCF3 and 5% BSA for OAS1B. Membranes were incubated with either anti-OAS1 antibody at 4°C for at least 16 hours or with anti-ABCF3 antibody for one hour at room temperature. Rabbit anti-ABCF3 polyclonal antibody (Bethyl Laboratories,

Inc.) was diluted 1:2000 with 0.2% non-fat dry milk in 1x TTBS (1% Tween 20 in 20 mM Tris, 500 mM NaCl, pH 7.5). Rabbit anti-OAS1 polyclonal antibody (Abcam Inc.) was diluted to 1:500 with 1% BSA in 1x TTBS. Secondary anti-rabbit goat IgG antibodies obtained from Bio-Rad were diluted to 1:3000 in 0.2% non-fat dry milk in 1x TTBS for ABCF3 or 1% BSA in 1x TTBS for OAS1B detection.

2.4.5 Densitometric Scanning and Quantification

The nitrocellulose membranes were scanned, and Multi-Guage V2.3 software was used for quantification of protein band intensity. The expression of ABCF3 or OAS1B under single expression conditions was designated as 1.0. A fold-change in expression of each protein under double expression conditions was calculated by dividing the amount of each protein in a double expression sample by the amount in a single expression sample from the same gel. Data from at least three independent experiments were combined to obtain average relative expression values.

2.4.6 Purification of GST-tagged ABCF3

E.coli Rosetta 2(DE3)pLysS cells containing pGEX plasmids were grown in 1 L of LB medium with ampicillin (100 μg/ml) at 37°C until mid-log phase was reached ($A_{600\ nm}$ =0.6) and then induced with 0.25 mM IPTG at 20°C for 16 hours. ABCF3 protein was purified after expression from the pGEX*f3* clone according to the manufacturer's instructions (GE Healthcare) with some modifications. The cell pellets were resuspended in 50 ml of Buffer A containing 10 mM DTT and complete protease inhibitor cocktail. The cells were broken by two passages through a French press followed by centrifugation as described above. The supernatant was mixed with 1.3 ml of washed glutathione sepharose (GE Healthcare) for 16 hours in a tube revolver at 10 rpm and then transferred to a 10 ml gravity-flow column. To obtain uncleaved GST-ABCF3 protein, the column was washed with three 10 column volumes of Buffer A with 1 mM DTT and eluted twice

with 1 ml of 10 mM glutathione in 50 mM Tris-HCl, pH 8.0 with 20% glycerol. To obtain ABCF3 without the GST tag, the column was washed five times with 10 column volumes, three times with Buffer A and two times with 1 x cleavage buffer (GE Healthcare) containing 20 % glycerol and 1 mM DTT (Buffer B). The washed sepharose was then removed from the column, mixed with 920 µl Buffer B and 80 µl of PreScission protease in an Eppendorf tube, and incubated on a tube revolver for 4 hours (10 rpm) at 4°C. The sepharose was then added back to the column, and the cleaved ABCF3 was eluted from the column twice with 1 ml of Buffer B. The protein concentration was determined using the DC™ assay (Bio-Rad), and aliquots were stored at -80°C until use.

2.4.7 Purification of His-tagged ABCF3

E.coli HMS174(DE3) cells transformed with pET28a DNA encoding the wild type or a mutant *abcf3* gene were grown in 1 L of LB medium with kanamycin (30 µg/ml) at 37°C until mid-log phase was reached ($A_{600\ nm}$ =0.6) and induced with 0.25 mM IPTG at 20°C for 16 hours. The cells were pelleted, the cell pellet was resuspended in 10 ml of Buffer A containing 1 mM DTT and complete protease inhibitor, and the cells were lysed with a French press followed by centrifugation as described above. The supernatant was then mixed with 2 ml of Ni-NTA agarose (previously washed with 40 ml of Buffer A containing 10 mM imidazole) in a closed 10 ml gravity-flow column on a tube revolver at 10 rpm for one hour at 4°C. The flow-through was collected and the column was washed with 50 ml of 30 mM imidazole and 1 ml of 100 mM imidazole. The ABCF3 protein was then eluted twice with 1 ml of Buffer A containing 200 mM imidazole. The two elutions were separately dialyzed against 500 ml of Buffer A overnight and again for two hours the next day before collection. Protein concentration was determined by the DC™ assay (Bio-Rad), and aliquots were stored at -80°C until use.

2.4.8 Site-directed mutagenesis of the Walker A or Walker B motifs of ABCF3

Site-directed mutagenesis of the *abcf3* gene was performed using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutations in the Walker A (K216A, K531A, K216R, and K531R) or Walker B (E353A, E636A, E353Q, and E636Q) domain of each NBD were created using the pET28a-*abcf3* plasmid as a template. Plasmid DNA with a single mutation in one NBD was used as the template to make a second mutation in the other NBD creating the double Walker A (K216A/K531A and K216R/K531R) or the double Walker B (E353A/E636A and E353Q/E636Q) mutants.

2.4.9 ATPase activity assay

The ATPase activity of 5 μg of purified wild type or mutant ABCF3 protein was determined using an ATPase activity assay, as described previously (44,87). The slope of the reaction was measured between 200-400 seconds and used to determine the ATPase activity in nmol/min/mg. Different concentrations of a ligand were added to 5 μg of purified ABCF3 in a 1 ml reaction volume.

2.4.10 Analysis of TNP-ATP binding to ABCF3

TNP-ATP binding assays were conducted with purified wild type or mutant ABCF3 proteins. TNP-ATP (0.1 μM to 20 μM) was added sequentially to 5 μM of ABCF3 in Buffer A in a total starting volume of 500 μl in each titration. The titrations were performed on an Alphascan-2 spectrofluorometer (Photon Technology Int., London, Ontario, Canada) with the following settings: 1.00 mm slit widths at 75 Watts with 403 nm excitation and 450-600 nm emission. To determine the increase in fluorescence resulting from TNP-ATP binding to the protein, values obtained from a negative control titration without any added ABCF3 were subtracted from the

respective fluorescence values obtained in reactions containing ABCF3. The fluorescence units obtained were then corrected for inner filter effects using Equation 1 (88),

$$F_{i,cor} = (F_i - F_B)(V_i/V_0) \times 10^{0.5b\ (A\lambda ex + A\lambda em)} \text{ (Equation 1)}$$

In Equation 1, $F_{i,cor}$ is the revised fluorescence intensity value based on inner filter effects; F_i corresponds to the preliminary fluorescence values; F_B is the fluorescence for the blank (no protein) titration at a given point; V_0 is the starting sample volume; V_i is the sample volume at a given point in the titration; *b* is the optical cell path-length measured in cm; and $A_{\lambda ex}$ is the absorbance at 403 nm excitation with $A_{\lambda em}$ the absorbance at emission wavelength 548 nm.

Percent increase in fluorescence was then obtained by using Equation 2,

$$\% \text{ Increase} = ((F_{i,cor} - F_{0,cor})/(F_{f,cor})) \times 100 \text{ (Equation 2)}$$

In Equation 2, $F_{i,cor}$ is the fluorescence intensity value corrected at a given point in the titration, $F_{0,cor}$ is the initial corrected fluorescence value for the initial titration value, and $F_{f,cor}$ is the final corrected fluorescence value for the titration. Nonlinear regression in GraphPad Prism 6 Software was used to analyze binding kinetics based on a single site, two site, or allosteric model for binding.

2.4.11 TNP-ATP Displacement assays

To determine if TNP-ATP binds to the nucleotide binding pocket(s) of ABCF3, titrations were performed with increasing concentrations of ATP (0.1-20 mM), ADP (0.1-20 mM) or AMP (0.1-20 mM). Briefly, 5 μM ABCF3 was mixed with 5 μM TNP-ATP and 10 mM $MgCl_2$ in 500 μl of Buffer A, and the reaction was incubated at room temperature for 5 minutes before starting the assay (55,56). Increasing amounts of nucleotide were then added to the sample and the fluorescence was monitored. For each experiment, a blank titration (sample prepared without

ABCF3) was also performed. The fluorescence values were corrected for inner filter effects according to Equation 1 above.

2.4.12 Intrinsic Trp Fluorescence Quenching Analysis

Intrinsic Trp fluorescence of ABCF3 was determined on an Alphascan-2 spectrofluorometer (Photon Technology Int., London, Ontario, Canada) with the following settings: 1.00 mm slit widths at 75 Watts with 295 nm excitation and 310-370 nm emission. Quenching of intrinsic fluorescence by ATP or ADP was then determined by titrating increasing amounts of nucleotide (5 μM-5 mM) into a 500 μl reaction volume containing Buffer A and 0.5 μM purified ABCF3 protein. Control titrations containing 10 μM NATA in the 500 μl reaction volume described above were also carried out with ATP or ADP to determine the degree of nonspecific quenching of tryptophan fluorescence. All fluorescence values obtained were corrected for inner filter effects with equation 1, using 295 nm excitation for $A_{\lambda ex}$ and 330 nm emission for $A_{\lambda em}$. Percent quenching was then obtained with Equation 3,

$$\% \text{ Quenching} = [(F_{0,cor} - F_{i,cor})/(F_{0,cor})] \times 100 \quad \text{(Equation 3)}$$

In Equation 3, $F_{i,cor}$ and $F_{0,cor}$ are the same values as described above. Kinetic analysis was performed using nonlinear regression with Graph Pad Prism 6 Software using one site or two site binding kinetics.

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3 CHAPTER II: ANTIBIOTIC RESISTANCE MECHANISMS IN BACTERIA: RELATIONSHIPS BETWEEN RESISTANCE DETERMINANTS OF ANTIBIOTIC PRODUCERS, ENVIRONMENTAL BACTERIA, AND CLINICAL PATHOGENS -A REVIEW

Emergence of antibiotic resistant pathogenic bacteria poses a serious public health challenge worldwide. However, antibiotic resistance genes are not confined to the clinic; instead they are widely prevalent in different bacterial populations in the environment. Therefore, to understand development of antibiotic resistance in pathogens, we need to consider important reservoirs of resistance genes, which may include determinants that confer self-resistance in antibiotic producing soil bacteria and genes encoding intrinsic resistance mechanisms present in all or most non-producer environmental bacteria. While the presence of resistance determinants in soil and environmental bacteria does not pose a threat to human health, their mobilization to new hosts and their expression under different contexts, for example their transfer to plasmids and integrons in pathogenic bacteria, can translate into a problem of huge proportions, as discussed in this review. Selective pressure brought about by human activities further results in enrichment of such determinants in bacterial populations. Thus, there is an urgent need to understand distribution of resistance determinants in bacterial populations, elucidate resistance mechanisms, and determine environmental factors that promote their dissemination. This comprehensive review describes the major known self-resistance mechanisms found in producer soil bacteria of the genus *Streptomyces* and explores the relationships between resistance determinants found in producer soil bacteria, non-producer environmental bacteria, and clinical isolates. Specific examples highlighting potential pathways by which pathogenic clinical isolates might acquire

these resistance determinants from soil and environmental bacteria are also discussed. Overall, this article provides a conceptual framework for understanding the complexity of the problem of emergence of antibiotic resistance in the clinic. Availability of such knowledge will allow researchers to build models for dissemination of resistance genes and for developing interventions to prevent recruitment of additional or novel genes into pathogens.

3.1 Introduction: A Brief Historical Perspective

Selman Waksman, a prominent researcher in the field of actinomycetes in the early part of the twentieth century, described the term antibiotic as a chemical compound generated from microorganisms that inhibits or destroys other microbes (Hopwood, 2007; Davies and Davies, 2010). Most antibiotics in use today originated from the phylum *Actinobacteria* with nearly 80% of actinobacterial-derived antibiotics produced by soil-dwelling bacteria of the genus *Streptomyces* (Barka et al., 2016). Before the discovery of natural antibiotics, synthetic compounds, including salvarsan, sulfa drugs and quinolones, were in use as chemotherapeutic agents (Aminov, 2010). Penicillin was the first natural antibiotic to be discovered accidentally by Alexander Fleming in 1928 when the *Penicillium* fungus contaminated a culture plate in his laboratory, however, penicillin was not developed for use until the late 1930s (Hopwood, 2007). Penicillin inhibits cell wall synthesis and was found to be very effective against Gram-positive but not against Gram-negative bacteria (due to the presence of the outer membrane) or the tubercle bacillus (because of the extra thick cell wall) (Hopwood, 2007). Following the discovery of penicillin by Fleming, other scientists, including Rene Dubos and Selman Waksman, started a deliberate search for antibacterial agents among soil microorganisms, including bacteria and fungi. It was soon realized that antibacterial activity was most often present in actinomycete cultures and less often in other bacteria or fungi. During this period, several antibiotics were discovered in the screens designed by these scientists but many of these were of little use in the clinic due to their toxicity in animals. The next biggest discovery came about in 1943, resulting in identification of streptomycin produced by *Streptomyces griseus*. Streptomycin inhibits protein synthesis by binding to the 30S subunit of the prokaryotic ribosome and was found to be effective not only

against Gram-negative bacteria but also against the tubercle bacillus (Hopwood, 2007). With the discovery of streptomycin, the golden age of antibiotic discovery and development (1940–1990) ensued. This involved efforts of many academic institutions and major pharmaceutical companies in the United States and other countries. Currently, antibiotics affecting almost every process in the bacterial cell are known. Based on their structure and mode of action, at least seven major groups of antibiotics have been described. These include β-lactams (inhibit cell wall synthesis), aminoglycosides (protein synthesis), macrolides (protein synthesis), tetracyclines (protein synthesis), daptomycin (cell membrane function), platensimycin (fatty acid biosynthesis), and glycopeptides (cell wall synthesis).

It is only natural that organisms which produce antibiotics should also contain self-resistance mechanisms against their own antibiotics. In addition, co-existence of producer and non-producer bacteria is also believed to have resulted in co-evolution of resistance mechanisms in non-producing environmental bacteria. Resistance determinants found in these two groups of bacteria have garnered significant attention in recent years because of their possible link with the emergence of resistance in pathogenic clinical isolates (Surette and Wright, 2017; Martinez, 2018). Indeed, with the global epidemic of antibiotic resistance unfolding before us, it is important to understand the origin of these determinants in pathogens. This review article provides an up-to-date understanding of the antibiotic self-resistance mechanisms found in producer soil bacteria of the genus *Streptomyces* and explores relationships between resistance determinants found in producer and non-producer soil and environmental bacteria and the clinical pathogenic bacteria. The topic of self-resistance in producer bacteria has never before been reviewed in its entirety, while antibiotic resistance mechanisms in clinical isolates have been extensively described (Munita and Arias, 2016). Therefore, resistance mechanisms of clinical isolates are not discussed

in detail in this article. Critical additional information about clinical isolates is, however, provided in a separate section following description of self-resistance in *Streptomyces*. These two sections were kept separate in this review because resistance mechanisms of producers and clinical isolates are currently at very different levels of understanding. In the last sections of this review, origins of resistance determinants in clinical strains and potential mechanisms for their mobilization are discussed. Although every attempt has been made to be inclusive of all available literature, the information on each topic addressed in this review is broad and constantly growing, therefore any omission is unintentional. Where possible, references to additional literature and review articles are provided for further reading.

3.2 Self-Resistance Mechanisms in Producer Organisms

Antibiotic producing bacteria contain a variety of sophisticated mechanisms for self-defense against their own antibiotics (Figure 3.1 and Table 3.1). Very often they contain multiple mechanisms simultaneously to ensure complete protection from the biologically active molecules produced by them. Interestingly, the genetic determinants for self-resistance are almost always clustered together with the antibiotic biosynthesis genes, and their expression is co-regulated (Mak et al., 2014). The following section highlights major biochemical categories of self-defense mechanisms found in producer organisms with specific examples provided for each category.

3.2.1 Antibiotic Modification or Degradation

Antibiotic modification is a commonly used strategy for rendering an antibiotic ineffective, especially in the case of aminoglycoside antibiotics (for example, kanamycin, gentamycin, and streptomycin), chloramphenicol, and β-lactams. A large number of aminoglycoside modification enzymes (AMEs), including *N*-acetyl transferases (AAC), *O*-phosphotransferases (APH), and *O*-adenyltransferases (ANT) that acetylate, phosphorylate, or adenylylate the aminoglycoside

antibiotic, respectively, are known to exist in producer bacteria. Although these enzymes were first identified in the producer *Streptomyces* species in the early 1970s, and they perform identical biochemical reactions to those seen in antibiotic resistant clinical strains (Walker and Walker, 1970; Benveniste and Davies, 1973), a direct correlation between synthesis of aminoglycosides and the presence of modification enzymes in producer *Streptomyces* is is not always evident. For example, some species may not produce antibiotics but still contain modification enzymes, and vice versa. One exception is streptomycin resistance, where a direct correlation between antibiotic synthesis and the role of modification enzymes in self-resistance has indeed been established. Streptomycin resistance in the producer *S. griseus* involves the function of the modification enzyme streptomycin 6-phosphotransferase that converts streptomycin to an inactive precursor streptomycin-6-phosphate. Streptomycin 6-phosphotransferase is the last enzyme in the biosynthetic pathway, and the expression of the gene encoding this enzyme is co-regulated with biosynthesis genes (Shinkawa et al., 1985; Mak et al., 2014).

Other than the example of streptomycin, the biological function of AMEs in the producer organisms has been a subject of unresolved debate for a long time. It has been speculated that these enzymes may not be directly involved in resistance in producers, but instead may perform other metabolic functions (Benveniste and Davies, 1973; Martinez, 2018). This claim is supported by comparative sequence analyses showing that the AMEs are quite diverse and are encoded by a large group of unrelated genes, thus suggesting that they might have originated by multiple convergent paths resulting in a similar function (Shaw et al., 1993). Other studies have also pointed out potential structural and sequence similarities between AMEs of producers and cellular metabolic enzymes, including similarity between APH and protein kinases and between AAC and protein acylases (Heinzel et al., 1988; Piepersberg et al., 1988; Davies and Wright, 1997),

implying that the modification enzymes might have been co-opted from housekeeping metabolic enzymes for antibiotic resistance. Thus many unanswered questions remain, which deserve a careful and systematic investigation. Future investigations should also determine if most aminoglycoside biosynthesis gene clusters found in producer *Streptomyces* contain genes for modification enzymes and whether these enzymes play a role in self-resistance.

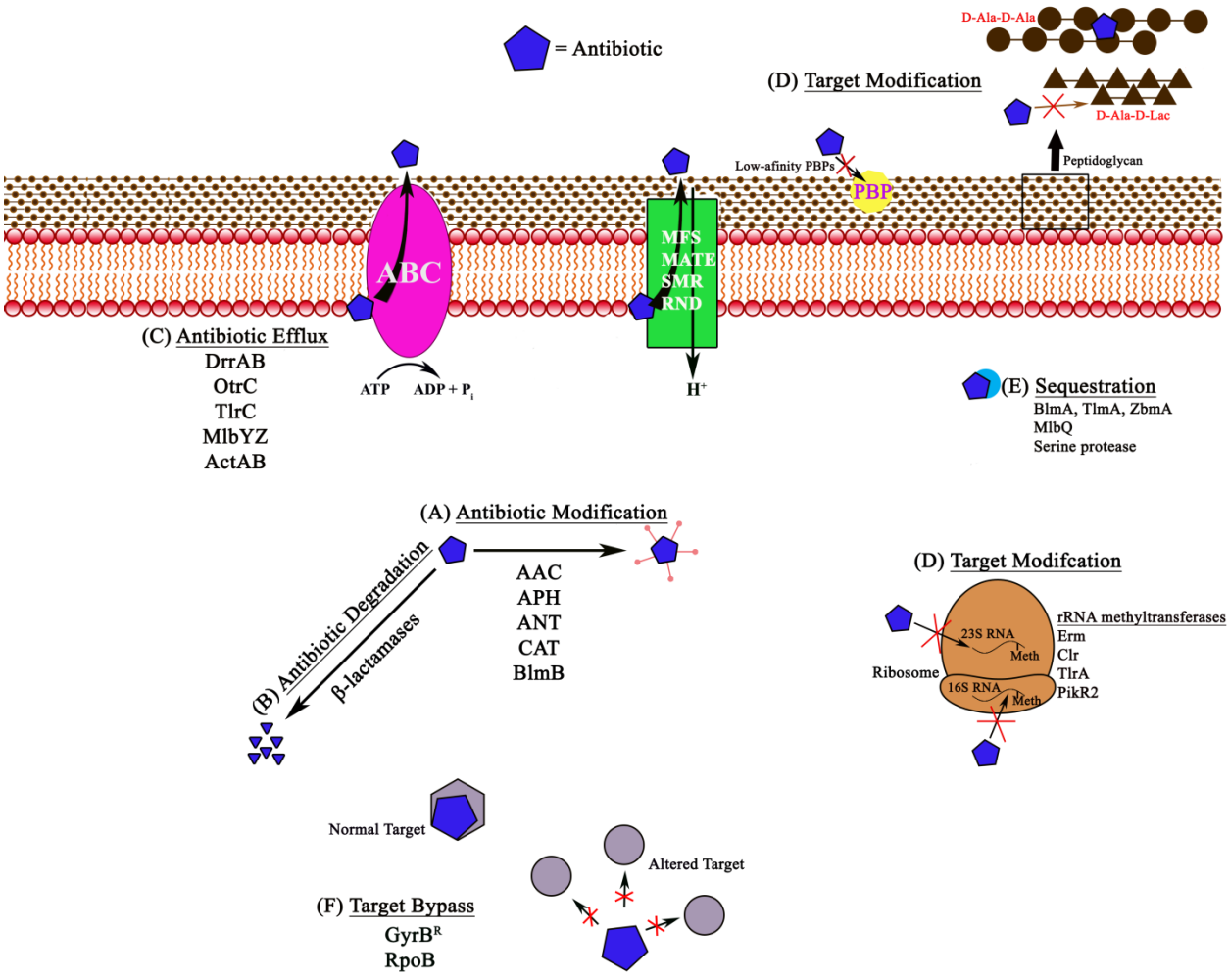

Figure 3.1: Schematic representation of different antibiotic resistance mechanisms in bacteria, shown with examples.

(A) Antibiotic modification involves the addition of acetyl, phosphate, or adenyl groups to aminoglycosides by N-acetyl transferases (AAC), O-phosphotransferases (APH), and O-adenyltransferases (ANT). Other examples include chloramphenicol acetyl transferases (CAT) and bleomycin N-acetyltransferases (BlmB). **(B)** Antibiotic degradation is observed with β-lactamases, which hydrolyze the antibiotic. **(C)** Antibiotic efflux pumps remove the antibiotic from the cell using energy from ATP hydrolysis in ABC pumps like DrrAB, OtrC, TlrC, and MlbYZ, or proton gradients in MFS, MATE, SMR, and RND family pumps. **(D)** Target modification includes various target alterations, such as 23S rRNA or 16S rRNA methylation, alterations in the peptidoglycan precursors (for example, in the case of glycopeptides), or synthesis of alternate low-affinity targets (PBPs) that reduce or completely block antibiotic (penicillins) from associating with the target. **(E)** Antibiotic sequestration involves proteins that can associate with the antibiotic and block them from reaching their targets. **(F)** Target bypass involves generation of additional antibiotic targets or subunits that are not susceptible to binding of the antibiotic. Meth, methylation.

Modification of the antibiotic as a mechanism for self-defense is also seen for other classes of antibiotics. For example, the bleomycin (BLM) family members [bleomycin (BLM), tallysomycin (TLM), phleomycin (PLM) and zorbamycin (ZBM)] are subject to acetylation. BLMs and TLMs are produced by *Streptomyces verticillus* and *Streptoalloteichus hindustanus*, respectively, and their biosynthesis gene clusters contain genes for *N*-acetyltransferases, BlmB and TlmB. These enzymes carry out acetylation of the metal-free forms of BLMs and TLMs, thus preventing correct formation of the metal-binding domain of these antibiotics (Coughlin et al., 2014). Finally, chloramphenicol is another antibiotic that can be acetylated by a large and widely distributed group of enzymes known as chloramphenicol acetyl transferases (CATs). Although

these enzymes have been shown to be very prevalent in clinical strains (Schwarz et al., 2004) and are also likely to be common in *Streptomyces,* only a few reports of identification of CAT enzymes from *Streptomyces* species are available (Murray et al., 1989).

In contrast to the modification of antibiotics described above, resistance to β-lactam antibiotics is normally conferred by antibiotic-hydrolyzing enzymes known as β-lactamases. These enzymes are widespread among *Streptomyces,* and, together with similar enzymes found in pathogenic and non-pathogenic bacteria, they constitute the 'β-lactamase superfamily' of proteins (Sattler et al., 2015; Ogawara, 2016b). β-lactamases are generally grouped into four classes (A,B,C,D) based on their amino acid sequence and use of a catalytic serine or zinc ion (King et al., 2016). In a recent phylogenetic screen conducted by Ogawara, it was found that diverse β-lactamases belonging to classes A, B, and C exist in many *Streptomyces* species. However, a clear relationship between the level of β-lactamases and the degree of resistance to β-lactam antibiotics in these species has not been established (Ogawara, 2016b). This is due to the fact that most *Streptomyces* species produce β-lactamases constitutively, and their production is not related to resistance or synthesis of β-lactams. As discussed previously for AMEs, *Streptomyces* β-lactamases also exhibit diverse species-specific properties, again suggesting convergent evolution from different proteins to perform the same function, i.e., hydrolysis of the β-lactam ring (Allen et al., 2009). The presence of β-lactamases in producers also presents an evolutionary conundrum – how can β-lactams and β-lactamases co-exist simultaneously in producer cells? Perhaps these enzymes play alternative cellular functions in *Streptomyces*, are expressed at low levels, or are expressed in a growth phase different from biosynthesis? Overall, therefore, it has been proposed that β-lactamases may not play an important role in resistance in *Streptomyces* species, which may

instead involve the function of low-affinity penicillin binding proteins (PBPs) (Ogawara, 2015) discussed in Section "Target Modification/Bypass/Protection Mechanisms" in this article.

Table 3.1: Antibiotic self-resistance mechanisms in producer bacteria.

Mechanism of Antibiotic Resistance	Selected Examples	Gene Location	Reference
Antibiotic Modification/ Degradation	**Aminoglycoside Modifying Enzymes (AME):AAC; APH; ANT** Streptomycin-6-phosphotransferase	Chromosome *S. griseus* (*smk)*	(Shinkawa et al., 1985; Mak et al., 2014)
	β-lactamases Class A,B,C β-Lactamases	Chromosome *Streptomyces* species	(Ogawara, 2016b)
Antibiotic Efflux	**ABC Transporter** DrrAB (Dox) OtrC (oxytetracycline)	Chromosome *S. peucetius (drr*AB*)* *S. rimosus (otr*C*)*	(Yu et al., 2012; Li et al., 2014)
	MFS Transporter OtrB (oxytetracycline) Mfs1 (natamycin)	Chromsome *S. rimosus* (*otr*B) *S. chattanoogensis* (*mfs1*)	(Ohnuki et al., 1985; Reynes et al., 1988; Wang et al., 2017)
Antibiotic Sequestration by Special Proteins	**Sequestration** TlmA, BlmA, ZbmA (bleomycin)	Chromosome *S. hindustanus* (*tlm*A); *S. verticillus (blm*A*); S. flavoviridis (zbm*A*)*	(Gatignol et al., 1988; Sugiyama et al., 1994; Rudolf et al., 2015)
Antibiotic Target Target Modification	**Low Affinity Penicillin-binding proteins (PBP)** Class A Class B	Chromosome *Streptomyces* species	(Ogawara, 2015; 2016a)
	Peptidoglycan remodeling (Glycopeptides) $VanH_{st}$, DdlM, $VanX_{st}$ $VanH_{aov}$, DdlN, $VanX_{aov}$	Chromosome *S. toyocaensis* (*van*$H_{st,}$ *ddl*M, *van*X_{st}); *A. orientalis* (*van*H_{aov}, *ddl*N, *van*X_{aov})	(Marshall et al., 1998; Binda et al., 2014)
	23S rRNA Methylation (MLS) Clr, PikR1, PikR2	Chromosome *S. caelestis* (*clr*) *S. venezuelae* (*pik*R1, *pik*R2)	(Calcutt and Cundliffe, 1990; Almutairi et al., 2015)
	16S rRNA Methylation (Aminoglycosides) PCT, Sgm methylase	Chromosome *S. pactum* (*pct*) *M. zionesis* (*sgm*)	(Ballesta and Cundliffe, 1991; Kojic et al., 1992)
Antibiotic Target Bypass	**DNA gyrase subunit B** (novobiocin)	Chromosome *S. sphaeroides (gyrB*^{R}*)*	(Schmutz et al., 2003)
Antibiotic Target Protection	**Antibiotic Removal** DrrC (Dox) OtrA (oxytetracycline)	Chromosome *S. peucetius* (*drr*C) *S. rimosus* (*otr*A)	(Doyle et al., 1991; Mak et al., 2014; Prija and Prasad, 2017)

AAC- N-acetyl transferases; APH- O-phosphotransferases; ANT- O-adenyltransferases; ABC-ATP-binding cassette superfamily; MFS- major facilitator superfamily; Dox- doxorubicin; MLS-

macrolides, lincosamides, and streptogramins; PCT- pactamycin methylase; Sgm- sisomicin-gentamicin resistance methylase.

3.2.1 Antibiotic Efflux

Efflux of antibiotics is another commonly used mechanism for self-resistance, although it usually occurs in conjunction with other mechanisms, such as modification of the antibiotic or the target. The best studied example of antibiotic efflux among producers is found in *Streptomyces peucetius*, which produces two closely related anticancer antibiotics, daunorubicin (Dnr) and doxorubicin (Dox). These two antibiotics intercalate with DNA preventing further rounds of replication. Efflux of these antibiotics in *S. peucetius* occurs by an ABC (ATP Binding Cassette) family transporter DrrAB coded by the *drr*AB genes embedded within the gene cluster responsible for biosynthesis of these antibiotics (Guilfoile and Hutchinson, 1991). The DrrAB system has been studied in significant molecular and biochemical detail. The DrrAB pump is assembled from two subunits each of the ABC protein DrrA and the integral membrane protein DrrB. DrrA protein functions as the catalytic nucleotide binding domain (NBD). DrrB protein functions as the carrier protein and forms the transmembrane domain (TMD). In an *in vitro* assay using inverted membrane vesicles, the DrrAB proteins were shown to carry out efflux of Dox in ATP or GTP-dependent manner (Li et al., 2014). Because of the location of the *drr*AB genes in the Dox biosynthesis gene cluster, this system is considered to be a dedicated transporter of Dnr and Dox in *S. peucetius*. Interestingly, however, recent studies showed that DrrAB pump is a multidrug transporter with broad substrate specificity, and it can transport many previously known MDR (multidrug resistance) pump substrates such as ethidium bromide, Hoechst 33342, verapamil, and vinblastine, among others (Li et al., 2014). In this regard, the DrrAB system is similar to the

mammalian ABC multidrug transporter *P*-glycoprotein (Pgp), which is overexpressed in human cancer cells and is one of the major causes for failure of chemotherapy (Chufan et al., 2015). Recent studies showed that critical aromatic residues, contributed by multiple helices in DrrB, form part of a large (common) drug-binding pocket (Li et al., 2014; Brown et al., 2017). Mammalian Pgp also uses aromatic residues to provide flexibility in substrate recognition, suggesting a common origin for these proteins and an aromatic residue-based mechanism for polyspecificity that is conserved over large evolutionary distances (Chufan et al., 2015; Szewczyk et al., 2015).

Interestingly, OtrC found in oxytetracycline producer *Streptomyces rimosus* is another example of a self-resistance efflux system that exhibits multidrug specificity. Self-resistance in *S. rimosus* is conferred by two efflux proteins: OtrB (previously known as TetB) located in the biosynthesis cluster, and OtrC located outside of the cluster (Mak et al., 2014). OtrB belongs to the major facilitator superfamily (MFS) of transport proteins, but not much is known about its mechanism of action or substrate specificity (Ohnuki et al., 1985; Reynes et al., 1988; Mak et al., 2014). OtrC protein is an ABC family protein, and like DrrAB, it also confers resistance to multiple antibiotics and MDR substrates, including ampicillin, oxytetracycline, doxorubicin, ethidium bromide, ofloxacin and vancomycin (Yu et al., 2012; Mak et al., 2014). Interestingly, the DrrAB and OtrC systems are quite homologous and show high sequence conservation in the previously identified motifs, including the DEAD and the LDEVLF motifs of DrrA (Zhang et al., 2010, 2015) and the EAA-like motif in DrrB (Kaur et al., 2005; Yu et al., 2012), suggesting close evolutionary links between efflux systems of different producer organisms.

It might be expected that efflux systems found in producer organisms would be specific for the antibiotic that the system is dedicated for. Surprisingly, however, the two examples (DrrAB

and OtrC) discussed above suggest polyspecific drug recognition in these systems. This raises interesting questions. Why is a multidrug transporter needed in a producer organism? What is the origin of DrrAB-like polyspecific antibiotic and drug efflux systems? Are most efflux systems associated with biosynthetic gene clusters polyspecific? Did these systems evolve from possibly even more ancient broad-spectrum efflux systems that might have served as general defense mechanisms against toxins in environmental bacteria? That transporters involved in antibiotic resistance could have been repurposed from the general defense efflux systems has been suggested previously (Dantas and Sommer, 2012; Martinez, 2018). Such an origin could explain why these systems are multi-specific, and how they could be easily adapted by different producer organisms to transport individual antibiotics synthesized by them. Analysis of many additional efflux systems found in biosynthesis clusters of producer organisms is needed to begin to formulate clear answers to these questions.

Many other examples of ABC as well as MFS transporters used for conferring self-resistance in producer organisms to lantibiotic NAI-107, polyene macrolide natamycin, tylosin, or actinorhodin are known (Rosteck et al., 1991; Xu et al., 2012; Mak et al., 2014; Pozzi et al., 2016; Wang et al., 2017). However, their molecular mechanisms and substrate specificities have not yet been elucidated.

3.2.1 Antibiotic Sequestration

Sequestration involves the function of drug-binding proteins, which prevent the antibiotic from reaching its target. In producers of the bleomycin family of antibiotics, the primary mechanism of resistance involves sequestration of the metal-bound or the metal-free antibiotic (Sugiyama and Kumagai, 2002) by binding proteins TlmA, BlmA, and ZbmA in *S. hindustanus* ATCC 31158 (Gatignol et al., 1988), *S. verticillus* (Sugiyama et al., 1994, 1995),

and *Streptomyces flavoviridis*, respectively (Rudolf et al., 2015). Each bleomycin-family producer member has one or more genes related to ABC transporters in their biosynthesis clusters (Du et al., 2000; Tao et al., 2007; Galm et al., 2009), which may be used to remove the antibiotics bound to binding proteins. For additional examples, see references (Sheldon et al., 1997, 1999; Pozzi et al., 2016).

3.2.1 Target Modification/Bypass/Protection Mechanisms

Target modification acts as a self-resistance mechanism against several classes of antibiotics, including β-lactams, glycopeptides, macrolides, lincosamides, and streptogramins (MLS), and aminoglycosides. The β-lactam antibiotic has a similar structure to PBP substrates (peptidoglycan precursors), thus allowing the antibiotic to associate and cause acylation of the active site serine resulting in its inhibition (Yeats et al., 2002). The producer *Streptomyces* species, despite being Gram-positive, are highly resistant to penicillins, which is due to either overproduction of PBPs or synthesis of low-affinity PBPs (Ogawara, 2015). Three classes of PBPs (A, B, and C) are found in bacteria (Ogawara, 2015). Analysis of the biosynthesis clusters of β-lactam producing bacteria showed that they often contain genes for PBPs, suggesting their role in self-resistance (Liras and Martin, 2006; Ogawara, 2015). Interestingly, *Streptomyces* species contain on average more than 10 PBPs, including both Classes A and B, a number much greater than found in other *Actinobacteria*. Some of these PBPs indeed have low affinity for β-lactams most likely due to the absence of a serine/threonine protein kinase domain (STPK) (renamed PASTA) that binds β-lactams (Ogawara and Horikawa, 1980; Nakazawa et al., 1981; Coque et al., 1993; Paradkar et al., 1996; Yeats et al., 2002; Ishida et al., 2006; Ogawara, 2016a).

Glycopeptides, such as vancomycin and teicoplanin, inhibit cell wall transpeptidation and transglycosylation by associating with peptidoglycan precursors (D-Ala-D-Ala) (Binda et al.,

2014). Antibiotic resistance results from a change in the peptidoglycan precursor from D-Ala-D-Ala to D-Ala-D-Lac or D-Ala-D-Ser, which has a 1000- and 6-fold reduction in affinity for the glycopeptides, respectively (Bugg et al., 1991; Billot-Klein et al., 1994). Genes conferring vancomycin resistance were initially identified in clinical strains, with the *van*A cluster (*van*HAX) on the transposon Tn*1546* being the most commonly seen. Some systems also use VanY, a D,D-carboxypeptidase to produce tetrapeptides incapable of glycopeptide binding (Binda et al., 2014). Related core *van*HAX clusters have been found in producer organisms, suggesting an evolutionary relatedness of resistance within producers and pathogens (Marshall et al., 1997, 1998). The examples include similar *van*H (Marshall et al., 1998), *van*A (Marshall and Wright, 1997, 1998), and *van*X (Lessard et al., 1998) sequences in the glycopeptide producers *Streptomyces toyocaensis* NRRL 15009 and *Amycolatopsis orientalis*. Variants on the core cluster are also reported (Schaberle et al., 2011; Binda et al., 2012; Marcone et al., 2014; Frasch et al., 2015). Other glycopeptide producers, without an obvious *van*HAX cluster, may have currently unidentified or poorly understood *van* resistance genes, such as *vanJ/sta*P (Hong et al., 2004; Novotna et al., 2012) and *van*K (Hong et al., 2005).

Target modification is also seen for MLS antibiotics, which bind to the 50S ribosomal subunit. This mechanism involves methylation of 23S rRNA at residue A-2058 by 23S rRNA methyltransferases (Douthwaite et al., 2004). Monomethylation (MLS type I) typically provides moderate level of resistance, while dimethylation (MLS type II) provides strong resistance (Fyfe et al., 2016). For further information on MLS resistance mechanisms, see reviews (Matsuoka and Sasaki, 2004; Mast and Wohlleben, 2014; Spizek and Rezanka, 2017). Finally, resistance against aminoglycosides by target modification uses 16S rRNA methyltransferases, which methylate at

residue A1408 or G1405 (Shakil et al., 2008). This mechanism for self-resistance may work in conjunction with the AMEs, which were described earlier.

Other resistance mechanisms bypass the original target by producing additional low affinity targets. Examples include synthesis of additional B subunit of DNA gyrase for novobiocin resistance, alternate resistant RNA polymerase for rifamycin resistance, or an alternate fatty acid synthase for resistance to platensimycin (Blanco et al., 1984; Thiara and Cundliffe, 1988, 1989; Schmutz et al., 2003; Sanchez-Hidalgo et al., 2010; Peterson et al., 2014). Antibiotic removal from the target site provides another protective resistance mechanism. In *S. peucetius*, DrrC removes intercalated daunorubicin/doxorubicin from DNA resulting in normal transcription and replication (Prija and Prasad, 2017). In *S. rimosus*, the antibiotic oxytetracycline is removed by OtrA from the ribosome (Doyle et al., 1991; Mak et al., 2014).

3.3 Multiplicity of Resistance Mechanisms in Producer Organisms

Most producer organisms contain several mechanisms for self-resistance. For example, *S. peucetius* relies on DrrAB to efflux doxorubicin (Li et al., 2014; Brown et al., 2017), DrrC to remove the antibiotic from its target DNA (Prija and Prasad, 2017), and DrrD is possibly used to modify the antibiotic to an inactive form (Karuppasamy et al., 2015). In addition, there is also a serine protease capable of sequestering daunorubicin to prevent its re-entry into the cell following efflux (Dubey et al., 2014). Other examples of producers containing several mechanisms for self-resistance include the following: *Microbispora* ATCC PTA-5024 contains both an efflux pump (MlbJYZ) and a sequestration protein (MlbQ) to protect against NAI-107 (Pozzi et al., 2016); *S. rimosus* has an ABC multi-drug efflux pump (OtrC) (Yu et al., 2012) and an MFS pump (OtrB) for efflux of oxytetracycline (Mak et al., 2014) along with OtrA to protect the ribosome by antibiotic removal (Doyle et al., 1991); *S. fradiae* contains several gene products (TlrA, TlrB, and

TlrD) that modify the ribosome to prevent tylosin binding and uses TlrC for efflux (Mak et al., 2014); and *S. chattanoogensis* L10 contains several different efflux pumps for resistance against natamycin (Wang et al., 2017).

3.4 Development of Antibiotic Resistance in Clinical Isolates

Discovery of antibiotics and their development for treatment of infectious diseases is the biggest success story in the history of chemotherapy. However, widespread and indiscriminate use of antibiotics in the last 70 years has led to selection of resistant strains to every antibiotic that has been introduced so far. With the very first antimicrobial agents, such as sulfonamides, resistance was observed soon after in the late 1930s (Davies and Davies, 2010). Even before the widespread use of penicillin in clinical practice, penicillinase was discovered in 1940 in *Staphylococcus aureus* and *Streptococcus pneumoniae* providing evidence that the resistance mechanisms against penicillin were already present in the natural environment (Davies and Davies, 2010; Ogawara, 2016b). Similarly, after the introduction of methicillin (a semi-synthetic penicillin) to treat penicillin-resistant *S. aureus* infections, resistance was once again observed in strains now referred to as MRSA (Methicillin-resistant *Staphylococcus aureus*) (Davies and Davies, 2010). These observations suggest that the use of each and every antibiotic sooner or later results in appearance of resistant strains. This is a testament to the extreme malleability and plasticity of bacterial genomes and their vast potential for adaptability. A high rate of spontaneous mutations and widely prevalent DNA exchange mechanisms in bacteria are critical contributors to the emergence of this phenomenon. According to the Centers for Disease Control and Prevention, antibiotic resistance leads to 23,000 deaths annually in the US alone. Recently, the development of MDR and XDR (extremely drug resistant) strains of *Mycobacterium tuberculosis, S. aureus,* and *Acinetobacter baumannii* have become a cause for serious concern, leaving limited options for the treatment of

infectious pathogens carrying these resistance mechanisms. These strains are commonly referred to as ‘superbugs,’ which can be normal human commensal flora that have acquired antibiotic resistance and increased virulence, such as MRSA strains of *S. aureus* and vancomycin resistant enterococci (VRE), or intrinsically resistant environmental bacteria that can become opportunistic pathogens, such as *Pseudomonas aeruginosa* and *A. baumannii* (Wright, 2007; Miller et al., 2014).

3.5 Mechanisms of Antibiotic Resistance in Clinical Isolates

3.5.1 Intrinsic vs. Acquired Resistance

Intrinsic antibiotic mechanisms are normally chromosome-encoded and include non-specific efflux pumps (which likely evolved as a general response to environmental toxins), antibiotic inactivating enzymes, or mechanisms that serve as permeability barriers (Fajardo et al., 2008; Cox and Wright, 2013). These mechanisms are fixed in the core genetic make-up of an organism. A well-studied example of an intrinsic resistance system is the AcrAB/TolC efflux pump in *Escherichia coli*, which has a very broad substrate specificity and can export different classes of antibiotics, dyes, detergents, and disinfectants (Nikaido and Takatsuka, 2009). Vancomycin resistance in *E. coli* and other Gram-negative bacteria provides another example of intrinsic resistance, which results from the permeability barrier imposed by the outer membrane (Arthur and Courvalin, 1993). Although intrinsic mechanisms confer low level antibiotic resistance in the original host, normal commensal flora or environmental bacteria containing intrinsic mechanisms can become opportunistic pathogens in immunocompromised patients (Wright, 2007). The acquired resistance mechanisms, on the other hand, are generally obtained by horizontal gene transfer (HGT, described later) and include plasmid-encoded specific efflux pumps (such as TetK and TetL of *S. aureus*) and enzymes that can modify the antibiotic or the

target of the antibiotic (Bismuth et al., 1990; van Hoek et al., 2011). These mechanisms pose a more serious threat to human health because of a change in the context of the resistance determinant from chromosomal to plasmid-mediated, resulting in their enhanced expression and dissemination (Dantas and Sommer, 2012; Martinez, 2018). A well-documented example of such a phenomenon is mobilization of the chromosomal β-lactamase gene *amp*C to a plasmid resulting in its worldwide dissemination (Dantas and Sommer, 2012).

3.5.2 Distribution and Function of Resistance Determinants in Clinical Pathogens

Interestingly, the biochemical mechanisms of resistance in clinical isolates are very similar to those found in producer organisms. Moreover, the resistance genes belong to the same functional families as seen in the producers (Benveniste and Davies, 1973; Marshall et al., 1998; Forsberg et al., 2012). However, the distribution, expression, and genetic context of resistance determinants in clinical strains are strikingly different. For example, resistance elements found in producer organisms are embedded in the biosynthesis gene clusters, while in clinical strains they are most often located on plasmids and transposons. For human health reasons, a lot more attention has been given to understanding the molecular and biochemical basis of antibiotic resistance in clinical isolates, and a large number of excellent reviews have been written on this topic (Blair et al., 2015; Chang et al., 2015; Munita and Arias, 2016). Therefore, the section below provides only relevant additional information about each resistance mechanism in clinical strains, allowing the reader to compare and contrast our understanding of these determinants in clinical strains vs. the producer organisms while providing a more complete picture of the field of antibiotic resistance. Where available, examples of antibiotic resistance genes/mechanisms in non-producing environmental bacteria are also provided, and their possible relationships with determinants in clinical strains are discussed (Table 3.2).

3.5.2.1 Antibiotic Modification

As seen in producers, antibiotic modification is commonly used as a resistance mechanism for aminoglycosides in pathogenic strains. Multiple types of AMEs (~100), including a fusion enzyme containing both AAC and APH activities, have been identified in both Gram-positive and Gram-negative bacteria (Schwarz et al., 2004; Ramirez and Tolmasky, 2010), and a detailed nomenclature has been developed (Ramirez and Tolmasky, 2010; Becker and Cooper, 2013). While these genes are commonly located on the mobile genetic elements (MGEs) in clinical bacteria, chromosomal determinants for AMEs have also been found in a large number of environmental bacteria, including *Providencia* and *Acinetobacter* species (Macinga and Rather, 1999; Yoon et al., 2014), which are considered to be the source of acquired determinants found on MGEs in pathogenic strains. Of the known AMEs, AACs are the most prevalent in clinical strains, and the AAC (6′) enzymes, which acetylate at the 6′ position of the aminoglycoside scaffold, have been studied in detail. In spite of the presence of a conserved fold, these enzymes exhibit significant sequence, structural, and functional diversity, again implying convergent evolution of these enzymes from distinct housekeeping cellular proteins (Stogios et al., 2017). Indeed, in the environmental bacteria *Providencia stuartii*, physiological function of the chromosomally encoded AAC(2′)-Ia enzyme is thought to be acetylation and recycling of peptidoglycan although it can also acetylate aminoglycosides (Macinga and Rather, 1999). Therefore, aminoglycosides may be ‘accidental’ substrates for these enzymes because of their similarity to cellular substrates containing amino sugars (Macinga and Rather, 1999). These studies further illustrate the plasticity of antibiotic modification enzymes (Fong et al., 2011; Stogios et al., 2017), as discussed previously for the producers. In addition to AMEs, multiple CAT enzymes have been identified in both Gram-

positive and Gram-negative bacteria, which have been extensively reviewed (Schwarz et al., 2004).

A third type of modification/degradation enzyme used by clinical bacterial strains is β-lactamase. While the role of β-lactamases in producer bacteria is still debatable, they are known to play a critical role in β-lactam resistance in Gram-negative clinical bacteria. Gram-positive bacteria instead prefer PBP-based resistance mechanisms, likely due to differences in the architecture of the cell wall/envelope between the two types of bacteria. More than 1000 β-lactamases have been identified from clinical isolates, and this number continues to grow because of the ever-new mutations in the active site allowing it to adapt to newer β-lactams. An example is the evolution of TEM-3, which can degrade 3rd generation cephalosporins, placing it into the category of ESBLs (Extended Spectrum β-lactamases) (Paterson and Bonomo, 2005), suggesting rapid evolution of β-lactamase genes in clinical strains. Most β-lactamase genes are carried on MGEs facilitating their rapid spread through populations; however, some β-lactamase genes are also found in chromosomes of members of the *Enterobacteriaceae* family where they are poorly expressed and function as silent genes. Once again, it is speculated that, as in the case of AMEs, β-lactamases may also perform dual functions, including housekeeping and antibiotic resistance (Martinez, 2018). An interesting set of studies indeed suggest that the biological function of β-lactamases may be peptidoglycan recycling (Wiedemann et al., 1998; Macinga and Rather, 1999), although their mobilization to a plasmid results in high expression and high levels of antibiotic resistance (Jacoby, 2009; Dantas and Sommer, 2012).

Table 3.2: Antibiotic resistance mechanisms in clinical isolates.

Mechanism of Resistance	Intrinsic Resistance	Gene Location	Ref.	Acquired Resistance	Gene Location	Ref.
Antibiotic Modification Degradation	**AME** AAC(2')-Ia	Chromosome *P. stuartii* (*aac(2')-Ia*)	(Macinga and Rather, 1999)	**AME** AAC(6')-Ib'	MGE *P.aeruginosa* (*aac(6')-Ib'* integron)	(Ramirez and Tolmasky, 2010)
	β-lactamase AmpC	Chromosome *E.coli* (bla_{AmpC})	(Jacoby, 2009)	**β-lactamase** TEM-3	MGE *K. pneumoniae* (bla_{TEM-3} plasmid)	(Paterson and Bonomo, 2005)
Antibiotic Efflux	**RND** AcrAB/TolC (MDR)	Chromosome *E. coli* (*acr*AB/*tol*C)	(Thanassi et al., 1997)	**SMR** QacC (MDR)	MGE *S. aureus* (*qac*C plasmid)	(Schindler and Kaatz, 2016)
	MFS NorA (MDR)	Chromosome *S. aureus* (*nor*A)	(Schindler and Kaatz, 2016)	**MFS** TetK, TetL (tetracycline)	MGE *S. aureus* (*tetK, tetL* plasmid)	(Bismuth et al., 1990; van Hoek et al., 2011)
Antibiotic Sequestration with Special Proteins	**Sequestration** Lipocalin (polymyxin B, rifampicin, norlfoxacin, ceftazidime)	Chromosome *B.cenocepacia (bcn*A)	(Sabnis et al., 2018)	**Sequestration** BLMS, BMLT (bleomycin)	MGE *S. aureus (ble* on plasmid) *E. coli* (*ble* on *Tn*5)	(Sugiyama et al., 1995; Kumagai et al., 1999)
Antibiotic Target Modification	**Low affinity PBP** PBP1	Chromosome *M. leprae* (*pon*1)	(Basu et al., 1996)	**Low affinity PBP** PBP2a	MGE *S. aureus* (*mec*A in SCCmec)	(Fishovitz et al., 2014)
	Peptidoglycan remodeling (GPAs) VanC, $VanXY_C$, $VanT_C$, $VanR_C$, $VanS_C$	Chromosome *E. gallinarum* (*van*C cluster)	(Binda et al., 2014; Miller et al., 2014)	**Peptidoglycan remodeling** (GPAs) VanRS, vanHAXYZ	MGE *E. faecalis* (*van*A cluster *Tn*1546 on plasmid)	(Binda et al., 2014; Miller et al., 2014)
	23S rRNA Methylation (MLS) ErmMT	Chromosome *M. tuberculosis* (*erm*MT)	(Buriankova et al., 2004)	**23S rRNA Methylation** (MLS) ErmC	MGE *S. aureus* (*erm*C plasmids)	(Roberts, 2008)
	16S rRNA Methylase (AGs) EfmM	Chromosome *E. faecium (efm*M)	(Galimand et al., 2011)	**16S rRNA Methylase** (AGs) ArmA	MGE *K. pneumoniae* (*arm*A on plasmid)	(Doi et al., 2016)
Antibiotic Target Bypass	**Overproduction** DHFR (TMP)	Chromosome *E. coli* (mutation in promoter of *dhfr*)	(Huovinen, 2001; Munita and Arias, 2016)	**Low Affinity** DHPS (sulfonamide)	Chromosome *N. meningitidis* (*dhps)* by transformation	(Radstrom et al., 1992)
Antibiotic Target Protection	**Antibiotic Removal** LsaA (lincosamide and streptogramin A)	Chromosome *E. faecalis* (*lsa*)	(Murina et al., 2018)	**Antibiotic Removal** TetO (tetracycline)	MGE *C. jejuni* (*tet*O) plasmid, transposon	(Munita and Arias, 2016)

AME- aminioglycoside modifying enzyme; AAC- N-acetyltransferase; MGE- mobile genetic element; RND- Resistance-Nodulation-Division; SMR- Small Multidrug Resistance; MFS- Major

Facilitator Superfamily; MDR- Multidrug resistance; PBP- penicillin-binding protein; GPAs- glycopeptide antibiotics; MLS- macrolides, lincosamides, and streptogramins; AGs- aminoglycosides; DHFR- dihydrofolate reductases; DHPS- dihydropteroic acid synthase; TMP- trimethoprim.

3.5.2.2 Antibiotic Efflux

The second major mechanism of antibiotic resistance in clinical strains involves decreased permeability and/or efflux of the antibiotic. Decreased permeability is important for Gram-negative bacteria because of the presence of the outer membrane, which forms a permeability barrier and offers an intrinsic mechanism for protection against hydrophilic antibiotics and other antimicrobial agents, such as vancomycin (Nikaido, 2003). Mutations in the porin genes and/or changes in their expression have been shown to further impact the susceptibility of Gram-negative bacteria to hydrophilic antibiotics (Li et al., 2012). In addition, many types of active efflux pumps have been described in Gram-positive and Gram-negative bacteria, which generally belong to one of the five families: ABC, MFS, RND (Resistance-Nodulation-Division), MATE (Multidrug and Toxin Extrusion), and SMR (Small Multidrug Resistance) (Sun et al., 2014; Schindler and Kaatz, 2016). Of these, only ABC proteins use ATP as a source of energy, while the other four families couple transport of substrates to ion gradients. Normally transport proteins carry out import or export of only one specific substrate (for example, Tet proteins belonging to the MFS family). However, examples of multidrug/polyspecific exporters have been found in each of these five families (Poole, 2005; Schindler and Kaatz, 2016), suggesting that polyspecificity is widely distributed and must be an ancient phenomenon.

Genes encoding antibiotic efflux pumps can be either intrinsic or acquired. Examples of intrinsic genes include *acr*AB/*tol*C in *E. coli*, *nor*A in *S. aureus,* and *lmr*A in *Lactococcus lactis*. Of these, the best understood system is the tripartite RND pump AcrAB/TolC. Although this system carries out efflux of a very broad spectrum of compounds, its biological function is believed to be export of bile salts in *Enterobacteriaceae* (Thanassi et al., 1997; Martinez, 2018). The RND pumps are unique in that they bridge the inner and outer membranes through a fusion protein (AcrA in this case) and bring about export of antibiotics from the inside to the outside in a single step. The acquired antibiotic efflux determinants, often found on MGEs in clinical isolates, are exemplified by many different types of *tet* genes (at least 22 have been identified) located on plasmids in both Gram-negative and Gram-positive bacteria (Roberts, 2005). Interestingly, RND pumps can act synergistically with the simple Tet pump proteins (MFS family), resulting in a significant increase in the minimum inhibitory concentration for tetracycline (Lee et al., 2000). This likely occurs when tetracycline exported to the periplasm by a Tet protein can be captured by the RND pump and exported to the outside (Nikaido and Takatsuka, 2009), illustrating how acquired resistance mechanisms can be augmented by the intrinsic mechanisms potentially resulting in major implications in the clinic.

3.5.2.3 Target Modification/Bypass/Protection

A large number of target replacement and protection mechanisms are also found in clinical isolates. The classical example of target modification is seen in MRSA strains where resistance to β-lactams is conferred by an exogenous PBP, known as PBP2a, whose transpeptidase domain is insensitive to the action of several different β-lactams. Acquisition of PBP2a facilitates bypass of the original sensitive target, however, since it does not contain the transglycosylase activity it functions together with the transglycosylase domain of the native PBP2 to perform cross-linking

reaction in the presence of β-lactams. PBP2a is coded by the *mec*A gene, which is located on a large MGE called SCC*mec* (Staphylococcal chromosomal cassette) in *S. aureus*. Many different types of SCC*mec* cassettes have been described, which contain varying numbers of accompanying resistance elements (Fishovitz et al., 2014; Liu et al., 2016). Another example of target modification is vancomycin resistance, which results from acquisition of the *van* gene cluster and is commonly a problem in enterococci (Miller et al., 2014). Of the many known types of *van* clusters, *van*A and *van*B, in particular, are a problem in clinical strains as they occur on MGEs. The similarities in the sequence and arrangement of *van* genes in producer and clinical strains suggest that they are evolutionarily linked.

Other target modification examples in clinical strains include point mutations or enzymatic alteration of the target (Munita and Arias, 2016). For examples of point mutations in the target, see (Hooper, 2002; Floss and Yu, 2005). Enzymatic alteration of the target is best understood in the case of macrolide resistance conferred by a large group of erythromycin ribosomal methylation (*erm*) genes. These enzymes methylate a specific adenine in the 23S rRNA (Weisblum, 1995). The *erm* genes in clinical strains are present on mobile genetic elements and are widespread among both Gram-positive and Gram-negative bacteria (Roberts, 2008). Significant similarities between the methylation enzymes found in the clinical isolates and the producers have been observed, suggesting a common ancestral origin (Uchiyama and Weisblum, 1985; Doi et al., 2016). Finally, known examples of target protection in clinical strains include the Tet(M) and Tet(O) proteins commonly encoded by genes located on MGEs in *S. aureus*. Interestingly, these proteins are homologous to the elongation factors EF-G and EF-Tu, and their binding to the ribosome facilitates removal of tetracycline in a GTP-ase activity-dependent manner (Burdett, 1996; Trieber et al., 1998).

Based on the discussion above, it is evident that our understanding of the distribution and function of resistance determinants in clinical isolates is much more advanced as compared to the producer organisms. It may also be concluded that many (or most) of the antibiotic resistance mechanisms in producers, and possibly all organisms, appear to have been repurposed from housekeeping/cellular functions or the intrinsic resistance mechanisms. Indeed, it is the incorporation of such determinants into MGEs in pathogens that poses a serious threat to human health.

3.6 Origin of Antibiotic Resistance in Clinical Isolates

Where do antibiotic resistance genes in the clinic come from? This question continues to puzzle scientists and clinicians. The idea that resistance genes in pathogens may be acquired from antibiotic producer organisms by horizontal transfer was originally proposed in the 1970s (Benveniste and Davies, 1973). It was based on the observation that the aminoglycoside-modifying enzymes found in actinomycetes exhibit biochemical activities similar to the enzymes found in pathogenic strains. Another striking example of a strong connection between antibiotic resistance genes in clinical isolates and those found in antibiotic producing bacteria is provided by the *van*HAX genes, which show considerable protein sequence similarity as well as a conserved arrangement and organization of genes within the cluster (Barna and Williams, 1984; Marshall et al., 1998).

Despite strong indications that transfer from producer organisms to the pathogenic strains might occur (Figure 3.2, Route 1) a direct link between producers and pathogens has, however, been hard to establish, and very rarely have the resistance genes of pathogens been tracked back to the producers. This is primarily due to the fact that resistance genes in producers show high sequence divergence and a very different G+C content as compared to determinants in pathogens

even when they use similar mechanisms (Forsman et al., 1990; Marshall et al., 1998). Altogether, these observations suggest an evolutionary link between determinants of producers and pathogens but not necessarily a direct recent gene transfer from the producers (Forsman et al., 1990; Marshall et al., 1998; Aminov and Mackie, 2007). Nevertheless, transfer from producers could have occurred a long time ago through a series of closely related carriers; for example, first transfer to closely related non-producing actinomycetes in the soil (Figure 3.2, Route 2A) and then finally to proteobacteria and distant pathogenic strains (Marshall et al., 1998) (Figure 3.2, Route 2B). The longer time horizon in this case could explain a very different G+C content in the two groups of organisms.

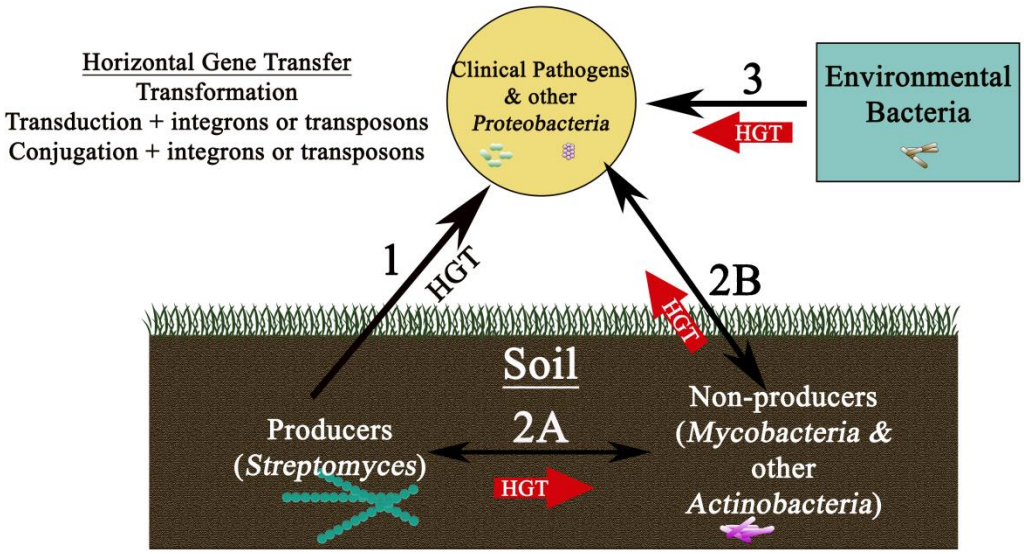

Figure 3.2: Schematic showing reservoirs of antibiotic resistance genes found in nature and various pathways for their movement to the clinic.

Transfer of resistance genes to clinical isolates could occur by a variety of routes (shown by arrows), each using horizontal gene transfer mechanisms potentially involving plasmids, integrons, or transposons. While direct transfer of resistance determinants from producers in the soil to clinical strains is possible (Route 1), a more likely route may first involve movement from

the producer soil bacteria to non-producer soil bacteria (for example *Mycobacterium* species) (Pang et al., 1994) (Route 2A), followed by transfer to clinical pathogens through several carriers (Route 2B). Another, possibly more important route, could involve direct transfer from environmental bacteria (found in bodies of water, aquaculture, livestock animals, wildlife, and plants) to clinical isolates (Route 3). Routes 2 and 3 are shown as thick red arrows, implying greater probability of these pathways for dissemination of resistance genes to clinical strains.

An alternative school of thought and a growing body of recent literature, however, now seem to suggest that resistance genes found in non-producer environmental bacteria may have played a more important role in shaping the evolution of antibiotic resistance in pathogens (Figure 3.2, Route 3) (Aminov and Mackie, 2007). Indeed, resistance genes are much more widespread in environmental non-pathogenic microbial populations than was originally believed (D'Costa et al., 2006; Nesme et al., 2014; Surette and Wright, 2017). In an interesting study, which tested 500 *Streptomyces* strains enriched and isolated from soil against 21 antibiotics (including natural, semisynthetic, synthetic as well as recently introduced antibiotics), surprisingly all strains were multidrug resistant to 7 or 8 of the 21 tested antibiotics (D'Costa et al., 2006), suggesting widespread resistance mechanisms among modern organisms. The genome sequence analyses carried out in recent years have also shown that not only are the intrinsic resistance mechanisms widely prevalent in all microbes (Fajardo et al., 2008; Cox and Wright, 2013), but that homologs of the resistance determinants of clinical isolates are commonly present in non-pathogenic Gram-positive and Gram-negative bacteria (Seoane and Garcia Lobo, 2000; Mukhtar et al., 2001; Sugantino and Roderick, 2002). Finally, there is also strong evidence showing that the antibiotic resistance gene sequences are ancient and predate the use of antibiotics (D'Costa et al.,

2011; Bhullar et al., 2012; Warinner et al., 2014; Perron et al., 2015; Kashuba et al., 2017). Analysis of microbial DNA isolated from the dental plaque of ancient human remains showed the existence of gene sequences homologous to those conferring resistance to β-lactams, aminoglycosides, macrolides, tetracycline, and bacitracin in clinical strains (Warinner et al., 2014; Olaitan and Rolain, 2016). In another study, metagenomic analysis of ancient DNA derived from 30,000-year-old permafrost showed the presence of homologs of *tet*M, *van*X, and *bla* genes (D'Costa et al., 2011). Interestingly, the *van*HAX cluster in permafrost DNA exhibited the same invariant organization as seen in modern vancomycin resistant isolates, confirming that these genes predate the use of antibiotics. Other similar studies showing prevalence of resistance determinants in ancient samples, or isolated caves, are also available (Bhullar et al., 2012; Perron et al., 2015; Kashuba et al., 2017). Together these findings suggest that there is a continuum of resistance genes present in the environmental, producer, and pathogenic organisms, leading to the concept of 'resistome' which is described as the collection of antibiotic resistance genes found in all microorganisms (Wright, 2007). Therefore, it is proposed that to get a full understanding of the origin of resistance, one must consider the pan-microbial genome consisting of antibiotic producers, pathogens, cryptic genes, and precursor genes (Wright, 2007; Nesme and Simonet, 2015).

Overall, it is safe to conclude that both producer and non-producing environmental organisms represent rich pools of resistance genes which could potentially be mobilized to the clinically relevant strains, leading to the question 'is the evidence for transfer of resistance determinants using any of the routes proposed in Figure 3.2 actually available'? Albeit limited in number, a few reports of direct genetic exchange from producer to non-producer organisms and from environmental organisms to clinical pathogens are indeed available. In one report, *otr*A

and *otr*B gene sequences, found in the oxytetracycline biosynthesis cluster in *Streptomyces*, were identified in mycobacteria variants (Pang et al., 1994). Mycobacterium is closely related to *Streptomyces*, and both are commonly found in the soil, therefore the transfer of *otr*A and *otr*B to mycobacteria suggests their role as potential carrier organisms in the soil. Interestingly, the same study also provided evidence for the presence of *S. aureus* tetracycline resistance genes Tet(K) and Tet(L) in *Streptomyces* and mycobacteria variants. The sequences isolated from these variants were almost identical to the *S. aureus* genes and had a G+C content of only 35% as compared to the 70% G+C content normally seen in *Streptomyces* and mycobacteria, which is a strong indication that these resistance elements originated from low G+C Gram-positive bacteria (Pang et al., 1994). This study therefore shows that resistance genes can move back and forth between producer and non-producer organisms providing support for Route 2A (Figure 3.2). In another study, bioinformatics analysis was used to obtain evidence for recent inter-phylum transfer of chloramphenicol and lincomycin efflux genes *cmx* and *lmrA* from *Actinobacteria* to *Proteobacteria* (Jiang et al., 2017), possibly also occurring through Route 2B, which may be followed by transfer of these genes to clinical isolates (Figure 3.2). The proposed mechanism for such inter-phylum exchange is discussed in (Jiang et al., 2017) and briefly described in Section "Role of HGT in Transfer of Antibiotic Resistance Genes" in this article.

The most compelling evidence of recent transfers from non-pathogenic environmental bacteria to clinical strains (Figure 3.2, Route 3) comes from three independent reports (Dantas and Sommer, 2012; Forsberg et al., 2012). First report showed that the CTX-M ESBL gene found on plasmids in pathogenic bacteria worldwide is almost identical to CTX-M gene found in the genome of non-pathogenic environmental *Kluyvera* species (Humeniuk et al., 2002; Canton and Coque,

2006), suggesting recent transfer of the gene to clinical strains. The second report shows that the quinolone resistance determinant *qnr* located on a conjugative plasmid in *Klebsiella*, originated from the genome of non-pathogenic environmental *Vibrio* and *Shewanella* species (Poirel et al., 2005). And yet another example provides evidence for transfer of the *aph*6 gene, which codes for Aph (3′)-VI amikacin modification enzyme, from the chromosome of the environmental *Acinetobacter guillouiae* to a plasmid in *A. baumannii* and then to members of *Enterobacteriaceae* family and to *Pseudomonas* species (Yoon et al., 2014). These examples provide definitive evidence of genetic transfer from environmental organisms and also illuminate how an intrinsic resistance gene located in the genome of a non-pathogenic organism can result in a pandemic when mobilized to a conjugative plasmid or a phage and transferred to a clinically relevant strain. Overall, these examples suggest that both producer and non-producer environmental bacteria play a role in dissemination of resistance genes although recent direct transfers to clinical strains seem to have mainly occurred from non-producer environmental bacteria.

3.7 Role of HGT in Transfer of Antibiotic Resistance Genes

Transfer of antibiotic resistance determinants between bacterial populations occurs by genetic exchange mechanisms involving transformation with free DNA, transduction by bacteriophages, or conjugation involving plasmids (Wright, 2007; Hu et al., 2017), collectively referred to as the HGT mechanisms. All three HGT mechanisms are widely used in nature, although certain species of bacteria tend to employ one mechanism more heavily over the others (Barlow, 2009). For example, streptococci can become naturally competent and thus participate effectively in transformation, whereas enterobacteria commonly use conjugative plasmids for exchange of genetic information. Transformation is best characterized in Gram-

positive *Streptococcus pneumoniae* and *Bacillus subtilis* although many Gram-negative bacteria also become competent (Johnston et al., 2014). The factors that control competence generally include the nutritional status of the bacterium (Claverys et al., 2006) and environmental stressors, such as antibiotics or DNA damaging agents (Prudhomme et al., 2006). Although the physiological role of transformation is still debated, its main purpose is believed to be DNA repair or genetic diversification to enhance adaptability (Johnston et al., 2014). Indeed, transformation seems to have played an important role in evolution of antibiotic resistance strains of *Streptococcus* and *Neisseria*. For example, it is thought that the persistence of penicillin resistance in *S. pneumoniae* may be related to the high frequency of natural transformation in this organism (Hoffman-Roberts et al., 2005). Transformation of *Neisseria gonorrhoeae* with DNA from resistant commensal *Neisseria flavescens* is believed to have resulted in generation of a mosaic *pen*A variant that confers resistance to β-lactams in clinical isolates (Spratt, 1988; Spratt et al., 1992). Mosaic variants of antibiotic resistance genes have also been reported in several *Streptococcus* species, implying the role of transformation in incorporating sections of foreign DNA (von Wintersdorff et al., 2016).

Transduction is believed to play a major role in evolution of resistance in *S. aureus*, although it has been shown to occur in many bacteria at a low frequency ranging between 10^{-6} and 10^{-9} transductants/plaque-forming-unit (Ubukata et al., 1975; Mazaheri Nezhad Fard et al., 2011; Varga et al., 2012). In *S. aureus*, which exhibits high strain variability and carries a large accessory genome consisting of phages, plasmids, transposons, genomic islands, and SCC_{mec} (most of which carry resistance genes), it is generally accepted that HGT in general, and transduction in particular, play a major role in antibiotic resistance gene transfer (Haaber et al., 2017). Indeed, moderate rates of transfer (about 10^{-5} or 10^{-6}) of genes for penicillinase, metallo β-

lactamase, and tetracycline resistance by transducing phages have been reported in *S. aureus* (Varga et al., 2012; Lee and Park, 2016; Varga et al., 2016). However, transduction of even the small SCC_{mecs} (20–25 kb in size) from MRSA strains of *S. aureus* to methicillin-sensitive strains was shown to occur at low frequencies (10^{-9} to 10^{-10}) (Scharn et al., 2013). Another study, which used qPCR to quantify *S. aureus* genes in viral particles, showed the presence of parts of the SCC_{mec} element (specifically *mec*A and *ccr*A1) in phage particles at relatively high frequency of about 10^{-4} (Maslanova et al., 2013). Quantitative studies, however, do not take into consideration the transmission capability of the particles, therefore they likely reflect an overestimation of the transduction frequency (Torres-Barcelo, 2018). Interestingly, other resistance and virulence genes of *S. aureus* associated with special MGEs referred to as PICIs (phage-induced chromosomal islands), which include SaPIs (*S. aureus* pathogenicity islands), are known to be transduced by bacteriophages at remarkably high frequencies approaching 10^{-1} (Chen and Novick, 2009; Penadés and Christie, 2015). These islands include many antibiotic resistance genes, suggesting that transduction may contribute significantly to variability and evolution of resistance in *S. aureus* (Novick et al., 2010). Interspecies and intergeneric transfer of SaPI elements has also been shown to occur between *S. aureus*, *S. epidermidis*, and even *Listeria monocytogenes*, showing a broader host range of staphylococcal phages (Maiques et al., 2007).

In general, however, because of the difficulty in detecting recombination events outside of the laboratory, the contribution of either transformation or transduction in transferring resistance genes in the clinic or the environment remains unclear. Nevertheless, certain environments considered to be hot-spots for genetic exchange, such as sewage and wastewater treatment plants, hospital effluents, aquaculture, agricultural and slaughterhouse waste, are prime locations for exchange events because of the high density of bacteria, phages, and plasmids in these settings

(Kenzaka et al., 2010; von Wintersdorff et al., 2016). In one study, qPCR analysis showed that *bla*$_{TEM}$, *bla*$_{CTX-M}$, and *mec*A were indeed present in phage particles isolated from sewage samples (Colomer-Lluch et al., 2014). Other reports showing the prevalence of phage carrying *bla*$_{TEM}$ and *bla*$_{CTX-M}$ genes in soil, water, and sewage are also available (Balcazar, 2014; Larranaga et al., 2018; Mohan Raj et al., 2018). When combined with high selection pressure in these environments, resulting from the presence of sub-inhibitory concentrations of antibiotics, metals, and toxic materials, which can lead to induction of competence (Prudhomme et al., 2006) as well as induction of prophages (Motlagh et al., 2015), it further enhances the possibility of HGT by these two mechanisms. Overall, these reports suggest that the original transfer of CTX-M from *Kluyvera* to the clinic pathogens, referred to in Section "Origin of Antibiotic Resistance in Clinical Isolates," might have been mediated by bacteriophages. Other settings suitable for genetic exchange via transduction also include the colonized human or animal host (McCarthy et al., 2014; Stanczak-Mrozek et al., 2015), gut microbiome (Modi et al., 2013), and biofilms (Resch et al., 2005). A recent report describing the phenomenon of auto-transduction in *S. aureus* provides further strong support for the important role of phages in delivering antibiotic resistance genes to the host bacteria (Haaber et al., 2016). Using *in vitro* and *in vivo* virulence model, this study by Haaber et al. (2016) demonstrates how phages released from a subpopulation of lysogenic cells can lyse other phage-sensitive cells in the same environment, recruit beneficial genes from the killed competitors, and reintroduce these genes into the remaining lysogenic host cells, resulting in genetic diversity.

Plasmid-mediated conjugation as a gene transfer mechanism is, however, still considered to be far more prevalent in disseminating resistance genes in nature than either transformation or transduction. Plasmids are capable of autonomous replication, and they carry genes for resistance

against all major classes of antibiotics. In fact, plasmids can carry a collection of resistance genes as part of transposons, thus simultaneously conferring resistance to several classes of antibiotics and metal ions (Nikaido, 2009). Moreover, they can transfer genes over long genetic distances to different species, genera, and even kingdoms depending on the host range of the plasmid. Using mathematical modeling analysis, one study recently showed that conjugation may be 1000-fold more common than transduction as a resistance gene transfer mechanism (Volkova et al., 2014). Since gene transfer by conjugation can be easily tracked by DNA sequencing and PCR-based approaches, there is sufficient evidence for its contribution to worldwide dissemination of antibiotic resistance determinants both in community and hospital environments (Carattoli, 2013). Some of the most successful known plasmids are the ones that have resulted in the spread of carbapenemase, *bla*$_{\text{CTX-M}}$ ESBL, and quinolone resistance genes among Gram-negative bacteria over very large geographical distances (Carattoli, 2013). In Gram-positive bacteria, other DNA elements, known as conjugative transposons or integrative conjugative elements (ICEs), can also mediate conjugation. These elements integrate into the chromosome but contain the ability to excise and transfer themselves by conjugation. ICEs often carry resistance genes, for example Tn*916* family members that encode tetracycline resistance (Roberts and Mullany, 2011). The known conditions for resistance gene transfer by conjugation include high density settings, such as the human or animal gut, biofilms, hospitals, and co-infection conditions (Weigel et al., 2003; Savage et al., 2013; Huddleston, 2014; Andersson and Hughes, 2017). Although some resistance determinants have been plasmid-associated for a long time (Barlow and Hall, 2002), others are mobilized to plasmids from chromosomes, and the rate at which these genes are being mobilized has increased since the widespread use of antibiotics about 70 years ago (Barlow et al., 2008). Another worrisome emerging trend is the clustering of antibiotic resistance genes on

plasmids, perhaps as a response to selective pressures in the environment. A well-characterized mechanism of clustering is provided by the *S. aureus* conjugative plasmid pSK41 that contains an insertion sequence IS*257*, which promotes capture of small resistance plasmids (Haaber et al., 2017).

All three HGT mechanisms are subject to limitations imposed by the host range of the incoming plasmid or the phage, the restriction modification systems of the host, ability to form cell-to-cell contacts, fitness cost of acquiring a new gene, as well as the ability of the incoming DNA to recombine with the host DNA (Thomas and Nielsen, 2005; Domingues et al., 2012). Further, the ability of a mobile genetic element to establish in a population also depends on whether it can replicate autonomously and therefore get vertically transmitted. The most successful conjugative plasmids, such as the incompatibility group IncP, have a broad host range (Davies and Davies, 2010), which facilitates their transfer to and maintenance in distantly related phyla (Klumper et al., 2015). The ability of MGEs or DNA to persist in the environment also determines success of HGT. For example, while cell-to-cell contact is essential for conjugation, it provides better protection to DNA. On the other hand, naked DNA is vulnerable to being degraded quickly, which reduces the time period during which it remains intact to successfully encounter a competent cell. DNA packed in a phage particle is more protected than naked DNA, although the narrow host range of a phage may determine if it will be in the gene pool long enough to infect a suitable host (von Wintersdorff et al., 2016).

In spite of the limitations, bacterial genome sequencing efforts have made it abundantly clear that the HGT mechanisms have had a major impact on evolution of bacterial populations (Nakamura et al., 2004; Andam et al., 2011; McDonald and Currie, 2017). Our knowledge of the actual steps and carriers involved in moving resistance genes from environmental and producer

organisms to the clinic, or from the chromosome to the MGEs, is, however, still rather limited. In each of the examples described in Section "Origin of Antibiotic Resistance in Clinical Isolates," exchange was facilitated by conjugative plasmids (Humeniuk et al., 2002; Poirel et al., 2005; Yoon et al., 2014) or by the presence of resistance genes on transposons (Brisson-Noel et al., 1988). It is not clear, however, why and how resistance genes are captured or transferred from chromosome to the plasmids. In addition to the role of insertion sequences and transposons, mobilization of resistance genes may also be greatly aided by the presence of integrons. While they are not self-mobile, they can be mobilized to plasmids or phages by transposons, thus gaining the ability to move between cells by HGT. Integrons typically contain three genetic elements, which include a gene for site-specific recombination (*Int*I), a site-specific recombination site (*att*I), and a promoter upstream of the *att*I site used for expression of the recruited gene cassette (often containing resistance determinants) (Domingues et al., 2012). Thus they are able to exchange and/or recruit gene cassettes by site-specific recombination between the *att*C site on the cassette and the *att*I site on the integron, or they can excise gene cassettes by site-specific recombination, therefore conferring the ability on the host to rearrange resistance and virulence determinants (Gillings, 2014). Class 1 integrons found on MGEs, in particular, are widely distributed in clinical settings and are often associated with carrying and spreading antibiotic resistance genes (Naas et al., 2001; Li et al., 2017). A rather large pool of circular gene cassettes containing the *att*C site and the promoter-less resistance determinants for almost all classes of antibiotics used clinically are also known to exist in bacteria (Partridge et al., 2009). These genes become functional after the cassettes are incorporated and expressed from the promoter sequence in the integron.

Recently, a novel 'carry-back' mechanism for inter-phylum exchange of genes was also proposed (Jiang et al., 2017). In this mechanism, conjugation mediated by a broad-host range

conjugative plasmid (Klumper et al., 2015) may transfer a carrier sequence of DNA (a fragment from a widely spread class 1 integron In4) from *Proteobacteria* to *Actinobacteria*, followed by recombination, resulting in actinobacterial DNA flanked by proteobacterial DNA. Dead actinobacteria cells would release the actinobacterial DNA flanked by proteobacterial DNA into the environment, and proteobacteria can take up this DNA by transformation and incorporate into their genome using homologous recombination. Using such a mechanism, *cmx* and *lmr*A genes are believed to have been recently transferred from *Actinobacteria* to *Proteobacteria* with the help of the broad-host range conjugative plasmids and integrons (Jiang et al., 2017). Once these genes are transferred to proteobacteria, it is easy to envision their transfer to pathogenic bacteria which also mostly belong to the phylum *Proteobacteria*. Indeed the *Proteobacterial* Cmx protein identified in clinical isolates was found to be 52% identical to the self-resistance protein from producer *S. venezuelae*, and the *cmx* gene was found to be 99% identical to genes from many non-*Streptomyces* actinobacteria, including *Corynebacterium* species, suggesting recent inter-phylum transfer from *Actinobacteria* to *Proteobacteria* following Route 2B.

3.8 Enrichment of Antibiotic Resistance Genes

By now it is well-recognized that the environment itself plays an important role in the acquisition of antibiotic resistance by pathogenic organisms. This process is envisioned to go through four stages: emergence of novel resistance genes, mobilization, transfer to pathogens, and dissemination. While emergence and mobilization events likely occur all the time, environmental factors, such as selective pressure, fitness cost, and dispersal, determine whether these events actually result in establishing novel genes in populations (Bengtsson-Palme et al., 2018). Of these, selection is perhaps the single most important factor which plays a critical role in maintenance of resistance genes/MGEs at each stage of the acquisition process described above. What creates

selective pressure strong enough to promote persistence and longevity of resistance genes? Antibiotic producers present one such scenario where resistance genes can be selected naturally in a competitive environment, thus preserving the pool of resistance genes in that niche (Laskaris et al., 2010). The most important source of selective pressure, however, is the widespread and indiscriminate usage of antibiotics by humans, which results in dominance of resistant and multiply resistant strains of bacteria not only among human pathogens but also in environments where human activities (such as antibiotic manufacturing facilities) result in pollution with antibiotics (Larsson, 2014). Other settings, considered to be hot-spots (described in section "Role of HGT in Transfer of Antibiotic Resistance Genes"), where human-associated and environmental bacteria co-exist, also provide significant opportunities for exchange of resistance genes as well as selection for resistance (Bengtsson-Palme et al., 2018). Such environments are ideal not only for transfer of resistance genes to pathogens, but they can also result in transfer of resistance from pathogens to environmental bacteria or opportunistic pathogens, resulting in persistence and possible reemergence of resistance genes in the future (Ashbolt et al., 2013; Martínez et al., 2014; Bengtsson-Palme et al., 2018). Recent studies have shown that antibiotic concentrations significantly below the minimum inhibitory concentration for sensitive bacteria can be selective (Gullberg et al., 2011, 2014). Moreover, other contaminants, such as heavy metals, can also co-select for antibiotic resistance (Pal et al., 2015; Andersson and Hughes, 2017).

There is indeed evidence that selective pressure caused by human activities in the last 70 years has resulted in a significant enrichment of resistance genes in bacterial populations. One study compared pre-antibiotic era microbes with modern environmental bacteria in archived soils collected from 1940 to 2008 in the Netherlands and showed that genes conferring resistance to tetracycline, erythromycin, and β-lactams increased in abundance over time (Knapp et al., 2010).

Interestingly, an increased rate of mobilization of β-lactamase genes from the chromosome to the plasmids was also reported (Barlow et al., 2008). A novel hypothesis advanced recently suggests that the use of antibiotics may provide a strong selection for 'capture' of antibiotic resistance genes by mobile genetic elements (including plasmids, transposons, and integrons) and acting as a strong force in shaping evolution of microorganisms (Gillings, 2014; Surette and Wright, 2017). Other reports also suggest that antibiotic selection promotes competence in *S. pneumonia* (Prudhomme et al., 2006), induction of prophages in *S. aureus* (Goerke et al., 2006), and enrichment of antibiotic resistance genes in phages present in the gut microbiome (Modi et al., 2013), all processes that could increase the rate of HGT. Interestingly, a more recent study showed that the ratio of transducing particles to virulent phages varies upon induction by sub-inhibitory concentrations of different antibiotics, suggesting that antibiotics affect packaging of genes into phage particles (Stanczak-Mrozek et al., 2017). Antibiotic exposure has also been shown to result in increased rates of mutations and recombination as well as an increase in integrase activity (Maiques et al., 2006; Lopez et al., 2007; Blazquez et al., 2012), thus compounding the multiple effects that excessive usage of antibiotics can have on emergence and enrichment of antibiotic resistance in bacterial populations. In conclusion, mitigation strategies focused on limiting selective pressure, for example by reducing unnecessary usage of antibiotics and avoiding settings which select for and promote persistence, are needed to prevent further recruitment of novel resistance genes into pathogens.

3.9 Conclusion, Research Gaps, and Future Directions

Antibiotic producing bacteria of the genus *Streptomyces* as well as non-pathogenic environmental bacteria are important reservoirs of antibiotic resistance determinants. These determinants may be transferred to clinical strains by a variety of HGT mechanisms, including

transformation of naturally competent bacteria, phages, and the use of conjugative plasmids, transposons, and integrons. Despite barriers to the exchange of genetic information between different genera of bacteria, widespread transfer of resistance genes from chromosomes of environmental and soil bacteria to the mobilizable elements in clinical isolates seems to have occurred. Indeed several examples of recent transfers from environmental bacteria to the clinical strains are available (Route 3, Figure 3.2); however, very limited evidence for recent direct transfer from producers to clinical strains has been obtained (Route 1, Figure 3.2). Nevertheless, transfer from producer bacteria to other actinomycetes in soil is possible (Route 2A), which could provide a pathway for further transfer of these determinants to proteobacterial clinical strains (Route 2B). Based on the available evidence, we conclude that Routes 2 and 3 are much more prevalent in nature as compared to Route 1 for transfer of resistance genes to pathogens.

To better understand factors that promote dissemination of resistance genes and to elucidate relationships between antibiotic resistance genes of producer, environmental, and pathogenic bacteria, new and improved strategies for sampling and screening of microbial populations and metagenomic libraries are needed. Moreover, better algorithms and the use of bioinformatics approaches for determining relationships between resistance determinants of different environmental niches will be highly beneficial. Additional genome sequencing data will also help fill the gaps in our knowledge of intermediate stages and carriers for mobilization. Indeed two databases, the Antibiotic Resistance Database (ARDB) and the Comprehensive Antibiotic Resistance Database (CARD), assembled in the last decade (Liu and Pop, 2009; McArthur et al., 2013), are expected to provide computational tools for the rapid prediction of antibiotic resistance genes and their targets in newly sequenced genomes and establish phylogenetic relationships. This was demonstrated in a recent bioinformatics study using these databases (Jiang et al., 2017). It is

expected that these bioinformatics tools will unify information on resistance genes and their products found in thousands of bacterial species isolated from the clinic or the environment as well as their associated mobile genetic elements and allow this information to be quickly mined by researchers in this field.

3.10 References

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4 GENERAL CONCLUSION

The spread and prevalence of flaviviruses, such as West Nile virus (WNV), continues to endanger public health [56]. Due to the unavailability of human vaccines and other therapies to prevent or treat this neurotropic virus, it is critical to identify host factors that reduce disease severity. The OAS/RNase L innate immunity pathway has been shown to have anti-flavivirus activity [58, 73]. The OAS/RNase L pathway responds to interferon signaling from the presence of dsRNA with OAS proteins producing 2-5A [60, 61]. The 2-5A then activates RNase L for cellular and viral ssRNA degradation [62]. The products of cleaved ssRNA may also act as ligands for RIG-I-like receptors leading to amplification of innate immunity signaling [58].

However, not all OAS1 proteins utilize the OAS/RNase L pathway to interfere with viral replication in host cells. The mouse OAS1B protein, one of eight mouse OAS1 proteins, was shown to produce a protein incapable of synthesizing 2-5A, even though it inhibits WNV replication inside host cells [63, 74]. Therefore, the mechanism of OAS1B-mediated flavivirus resistance is independent of the OAS/RNase L pathway. The mouse OAS1B protein localizes to the ER membrane through a TMD at its C terminus. Two OAS1B binding partners, ABCF3 and ORP1L, were previously identified in a yeast-two hybrid screen using a mouse brain library [55]. Association of both ABCF3 and ORP1L with OAS1B was further confirmed by both *in vitro* and *in vivo* co-immunoprecipitation assays [55]. Co-immunoprecipitation assays in WNV infected mammalian cells also showed that both the ABCF3-OAS1B-ORP1L complex and the viral NS3 helicase are associated with the ER. Co-localization of the two identified cellular binding partners with OAS1B was also demonstrated after co-transfection of the cDNA of each partner protein with OAS1B into BHK cells by IFA [55]. Additionally, ABCF3 knockdown experiments showed enhanced viral yields of WNV, but not of those of the non-flaviviruses, VSV and SINV, showing

that ABCF3 is involved in the flavivirus resistance. ORP1L knockdown experiments demonstrated reduced viral levels for both WNV and the tested non-flaviviruses, presumably because ORP1L knockdown dysregulates endosomes used by all of these viruses to enter host cells [55]. Due to the effect on the resistance phenotype previously seen in ABCF3 knockdown experiments, in Chapter 1 we focused on the biochemical characterization of ABCF3 to gain insights into its role in the OAS1B-mediated flavivirus resistance mechanism.

Purified ABCF3 protein showed basal ATPase activity of around 130 nmol/min/mg. To identify substrates of ABCF3, we tested the effect of known MDR drugs and various lipids on its ATPase activity. MDR drugs like Hoechst 33342, quinidine, verapamil, and vinblastine are transported by several ABC proteins and are known to modify their ATPase activity [22, 65-67]. However, the ATPase activity of purified ABCF3 was unaffected by these MDR drugs, suggesting no protein-drug interaction occurred. Many ABC proteins are also known to interact with and efflux lipids [11, 13, 15-17]. Since flaviviral infections modify the lipid content in cell membranes, including in the ER, which is the major site of lipid biosynthesis and OAS1B-ABCF3 complex localization [55, 68, 69, 75, 76], we next tested the effect of various lipids on ABCF3 ATPase activity. Our results showed that several lipids, including sphingosine, sphingomyelin, PAF, and LPC stimulated ABCF3 ATPase activity about three-fold or more compared to basal activity levels. Because LPC produced nearly four-fold stimulation of ABCF3 ATPase levels, we also examined the LPC-derived anti-cancer drugs miltefosine, edelfosine, and perifosine [77]. Miltefosine generated a biphasic response, with modest activity stimulation at low levels, and inhibition at high levels. The other alkyl ether lipids edelfosine and perifosine both inhibited ATPase activity. Other ligands, including lyso PAF, lysophosphatidylinositol (LPI), and cholesterol, also inhibited the activity of ABCF3. Overall, these results suggested that ABCF3

directly binds to various lipids and lipid-based drugs and is sensitive to small changes in lipid structure.

We also studied the nucleotide binding properties of wild type and mutant ABCF3 proteins using both tryptophan quenching and TNP-ATP binding analyses. Tryptophan quenching is commonly used to examine conformational changes resulting from ligand-protein interactions, which can be detected by fluorescence quenching [66, 67, 78]. We observed saturable quenching of tryptophan fluorescence when wild type ABCF3 was titrated with either ATP or ADP. The fluorescence data could only be fitted to single-site Michaelis-Menten kinetics, suggesting that ABCF3 only has one nucleotide binding pocket even though it contains two NBDs. Furthermore, conserved lysine to arginine mutations in the Walker A motifof each NBD alone or together (K216R, K531R, and K216R/K531R) showed no significant inhibitory effect on ATP binding to ABCF3, which may be due to the asymmetric distribution of tryptophan residues in ABCF3, as discussed in Chapter 1. Together these results suggested that tryptophan quenching analyses may not be suitable for analyzing ATP binding to ABCF3. Therefore, we further examined the nucleotide binding properties of ABCF3 using the fluorescent ATP analog TNP-ATP.

Association of TNP-ATP to the nucleotide-binding pocket in a protein leads to enhanced fluorescence in comparison to TNP-ATP in solution [67, 79-81]. In this study, titrations of purified ABCF3 with TNP-ATP demonstrated specific binding with a Hill coefficient of 1.8, suggesting the presence of two nucleotide binding pockets. Moreover, ABCF3 displayed allosteric sigmoidal binding kinetics, indicating that the two pockets of ABCF3 function in a cooperative manner. We further studied the role of each NBD by examining the effect of both conservative and non-conservative Walker A motif mutations on TNP-ATP binding. Each conservative single mutation (K216R or K531R) resulted in a lower TNP-ATP binding affinity, while the double

(K216R/K531R) mutant produced the strongest negative effect on affinity. Interestingly, the NBD2 mutant (K531R) showed a lower binding affinity than the NBD1 mutant (K216R), suggesting that the NBD2 of ABCF3 plays a more important role in nucleotide binding. As expected, the non-conservative Walker A lysine to alanine mutations (K216A, K531A, K216A/K531A), produced a much more drastic effect on TNP-ATP binding affinity. Moreover, TNP-ATP titrations carried out with the non-conservative mutant proteins showed incomplete saturation in each case, further confirming their drastic effect on the binding affinity. Altogether, these results implied that both NBDs are important for the function of ABCF3, although they contribute unequally to nucleotide binding.

To study the role of each NBD in ATP hydrolysis, we examined the effects of Walker A and B motif mutations on both basal and sphingosine-stimulated ATP hydrolysis. Our results showed differential effects of NBD1 and NBD2 mutations on ATPase activity. NBD1 mutations completely inhibited the basal activity of ABCF3, while the NBD2 mutations had little or no effect. Sphingosine showed a three-fold stimulation of the activity of wild type ABCF3. Interestingly, sphingosine also stimulated the ATPase activity of NBD1 mutant proteins but inhibited the ATPase activity of NBD2 mutant proteins. Based on these results and the known head-to-tail interaction of NBDs in other ABC proteins [26, 28], we generated a model for the nucleotide-binding pockets of ABCF3 (Chapter 1). In this model, pocket 1 is formed by an association of the Walker A and Walker B motifs of NBD1 with the ABC signature motif of NBD2, and pocket 2 is formed by an association of the Walker A and Walker B motifs of NBD2 with the ABC signature motif of NBD1 [26, 28]. Since proteins with mutations in NBD1 severely impacted the basal ATPase activity while proteins with mutations in NBD2 had little effect on basal activity, we propose that pocket 1 is the site of basal catalysis. We also propose that pocket 2 is the site of

ligand-stimulated ATPase activity because proteins with mutations in NBD1 still showed high sphingosine-stimulated ATPase activity, and proteins with mutations in NBD2 displayed inhibition of activity with sphingosine.

Interaction between ABCF3 and OAS1B was previously shown by a yeast two-hybrid assay, *in vitro* coimmunoprecipitation assay, and by *in vivo* coimmunoprecipitation assay, and co-localization was detected by IFA in mammalian cells [55]. In this study, we analyzed the interaction of ABCF3 with OAS1B in *E. coli* cells by using the pETDuet-1 system. The pETDuet-1 vector allows for the simultaneous expression of proteins to enable complex formation [70-72]. A complex of ABCF3 and OAS1B in bacterial membranes could provide a means for studying the possible transport of lipids and lipid-based drugs by this protein complex and facilitate purification of the complex for further biochemical analyses. We showed that the expression of OAS1B alone resulted in severe growth inhibition of *E. coli* cells, confirming that it is a membrane-embedded protein. Surprisingly, we found that co-expression with ABCF3 rescued the severe cell growth inhibition caused by expression of OAS1B, indirectly suggesting an interaction between these two proteins in bacterial cells. Co-expression of ABCF3 with OAS1B also resulted in significantly enhanced OAS1B levels in *E. coli* cells, suggesting protection of OAS1B from cellular proteolysis by interaction with ABCF3. However, the OAS1B protein strongly localized to inclusion bodies, rather than to the cell membrane. This is not surprising as membrane protein overexpression in bacteria typically results in toxic effects, proteolysis, and/or protein aggregation in inclusion bodies [82-89]. Future experiments will utilize this rescue of growth inhibition phenotype to identify and examine protein regions necessary for OAS1B interaction with ABCF3. Moreover, purification of OAS1B from inclusion bodiescould be optimized to obtain protein for further biochemical analyses [90, 91]. Future studies could also optimize localization of the ABCF3-

OAS1B protein complex to bacterial membranes by controlling temperature of expression and gene copy number to achieve lower levels of protein expression [92-94].

The mechanism by which the OAS1B-ABCF3 protein complex confers flaviviral resistance is unknown. Based on the results shown in this work, the protein complex may reduce flaviviral replication by enhanced levels of ATP hydrolysis. Our results showed that several lipids, including sphingosine and sphingomyelin, stimulated the ATPase activity of ABCF3. These lipids were previously shown to be increased in flavivirus-infected cells [69, 75, 76]. We also demonstrated that despite its inability to generate 2-5A [63], purified OAS1B contained a basal ATPase activity of about 90 nmol/min/mg. Since high ATP concentrations are required for efficient viral replication, the OAS1B-ABCF3 complex may thus limit the available ATP. The dengue virus NS3 helicase was previously shown to require large amounts of ATP to unwind dsRNA templates during flaviviral RNA synthesis [95]. Also, the viral polymerase NS5 requires large amounts of ATP for RNA synthesis [96]. In addition, lipid biosynthesis required for viral membrane reorganization also requires ATP [69]. Enhanced ATP hydrolysis by the OAS1B-ABCF3 complex could lower ATP levels at sites of viral replication and inhibit viral RNA production at any of these levels.

Chapter 2 of this dissertation presents a comprehensive review of the antibiotic resistance mechanisms found in producer soil bacteria and pathogenic bacteria found in clinical settings. In addition to other resistance mechanisms, the role of class 1, 2, and 3 ABC proteins in conferring antibiotic and multidrug resistance is discussed. This review also explores relationships between resistance genes found in producer, environmental, and pathogenic bacteria and discusses different horizontal gene transfer mechanisms that play a role in dissemination of these genes to the pathogenic bacteria.

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