XENOBIOLOGIC EFFLUX IN BACTERIA AND FUNGI: A GENOMICS UPDATE
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General Introduction

“……not enough to kill the streptococci but enough to educate them to resist penicillin.”

(Alexander Fleming, Nobel Prize lecture Dec 11, 1945). These prophetic words underscore the arms race that we find ourselves in today. Large populations and mutable genomes give microbes a profound capacity to respond to changing environmental conditions. The misuse of antibiotics in human health and agriculture has contributed to continuing microbial drug resistance. Thus 64 years later in 2009 we continue to battle microorganisms and strive to design novel and useful antimicrobial agents (1).

I. Bacterial Efflux Pumps

A. Types and substrates

The efflux of antibiotics was first discovered by Steward Levy and coworkers, in 1980, who were studying the mechanism of tetracycline resistance in *E. coli* (2). Since that time it has been demonstrated that a single efflux pump can provide resistance to multiple antibiotics (MDR efflux pumps). It has also been found that MDR-like transporters are highly abundant and ubiquitous in nature and represent, on average, more than 10% of the total number of transporters per organism (3).

Although MDR efflux pumps play an important role in the inherent resistance of bacteria to antibiotics, these pumps appear to be evolutionarily ancient transporters that have a wide variety of physiological functions, beyond antibiotic resistance, which contributes to adaptation to a wide variety of different environments (4-6).

Phylogenetically, bacterial antibiotic efflux pumps belong to one of five families (Fig. 1): 1) SMR or small multidrug resistance subfamily of the DNT (drug/metabolite transporters) superfamily (3, 7); 2) MATE or multi-antimicrobial toxic compound extrusion subfamily of the MOP (multidrug/oligosaccharidyl-lipid/polysaccharide flipases) superfamily (8, 9); 3) MFS or major facilitator superfamily (10); 4) RND or resistance/nodulation/division superfamily (11); and 5) ABC
or ATP-binding cassette superfamily (12). MDR transporters can also be classified into two main groups based on the mode of energy coupling for transport/efflux: 1) primary active transporters that belong to the ABC superfamily and utilize ATP hydrolysis to expel the drug from the cell; and 2) secondary transporters that utilize the proton motive force or ion gradient for drug expulsion. SMR, MATE, MFS and RND pumps are secondary transporters or antiporters. Other classifications have been proposed (13, 14) and several reviews have been dedicated to the classification and descriptions of MDR transporter families (15-22).

1. ABC Pumps

ABC (ATP binding cassette) transporters are found in all living organisms and are classified as primary active transporters that belong to the ATP binding cassette (ABC) superfamily and utilize the free energy of ATP hydrolysis to expel the drug from the cell. Historically the first characterized MDR transporters were members of the ABC superfamily of eukaryotic origin such as P-glycoprotein (23-25). Since that time, ABC pumps are also found in pathogenic fungi and parasitic protozoa where they impart resistance to antimicrobial drugs (26). The first bacterial ABC MDR pump (LmrA, Lactococcus lactis) was reported in 1996 (27). ABC pumps have been found to be widespread among bacteria (14) and appear to play an important role in drug resistance in some pathogenic bacteria such as Enterococcus faecalis and Streptococcus pneumoniae (28) (Table 1). Many of these MDR efflux pumps are homologues of the heterodimeric LmrCD pump of L. lactis.

ABC transporters have structural characteristics that set them apart from other efflux pumps. They usually have two similar halves, each containing two parts: a transmembrane domain (TMD) that is usually arranged into six transmembrane-spanning α-helices, and a nucleotide binding domain (NBD), also known as the ATP binding cassettes domains and contain the Walker A, Walker B and the ABC signature motifs (29). The NBDs are responsible for the binding and hydrolysis of ATP and hence the generation of the energy of the translocation of the substrate, while the TMDs form the translocation pathway for the transported substrates to cross the cytoplasmic membrane. In most cases a single protein contains a TMD-NBD-TMD-NBD structure
However, in bacterial transporters, a TMD fused to a NBD forms a “half-transporter” which homo- or hetrodimerizes with another “half-transporter” to form a functional full-size transporter (30), for example the homodimeric LmrA or heterodimeric LmrCD ABC pump of L. lactis. There are exceptions however, DrrA and DrrB found in Streptomyces peucetius, contain a single NBD or TMD domain respectively and are thought to function as a tetramer (31).

The generally accepted mechanism by which ABC transporters function is often explained by the “two-cylinder engine mechanism” (30, 32). In this model, drugs enter a high-affinity site in the TMDs of the ABC transporter from the cytosol side of the cytoplasmic membrane and then, upon the binding/hydrolysis of ATP (at the NBDs to provide the power stroke) and through conformational cycling, the substrate is occluded at the high affinity site and progresses to a low-affinity release site on the extracellular side of the membrane (Figure 2). As depicted in Figure 1, in some Gram-negative bacteria, ABC MDR efflux pumps have periplasmic accessory or membrane fusion proteins (MFPs). These MFPs interact with outer membrane channel or efflux proteins (OEPs) to form a tripartite pump that bridges both membranes of the Gram-negative envelope to mediate the extrusion of substrates from the cell such as the MacAB-TolC ABC MDR pump. MacA is a periplasmic protein of the MFP family, TolC is an outer membrane channel protein and MacB is a half-type ABC transporter with four putative TMD segments and an N-terminal NBD (34, 35). Table 1 lists examples of ABC MDR pumps found in bacteria and their substrates.

2. SMR Pumps

SMR (Small multi-drug resistance)-mediated multiple drug resistance is widespread among bacteria (16). These bacterial MDR efflux pumps are among the smallest known pumps and are made up of proteins that are typically 100-115 amino acid residues in length. SMR pumps have a 4-transmembrane α-helical topology (7) and a highly conserved residue, Glu14 (49, 50), that has been shown to be essential and directly involved in drug and proton binding (51, 52). It has been assumed that these pumps function as oligomeric complexes, perhaps as dimers, and that the Glu14 of both protomers in a dimer form a shared binding pocket (51, 53).
The genes encoding SMR pumps are found in a variety of plasmids from clinical isolates of *S. aureus* and other staphylococi (54-56) as well in the chromosome of many bacteria (57-59) (Table 2). The substrates of SMR pumps typically share similar physical properties but may differ in size and shape, and are almost exclusively monovalent hydrophobic cations.

The SMR pump, EmrE of *E. coli* is one of the better characterized of the SMR pumps (49, 52, 60). EmrE transports a diverse array of aromatic, positively charged substrates in exchange for protons (61). A model for its translocation cycle has been suggested in which the binding of the drug to its binding site (Glu14) deprotonates the Glu14 residues in the functional antiparallel dimer and causes for the transporter to undergo a conformational change in which the binding sites close behind the substrate, and opens in front of the substrate to expose it to the outer face of the cytoplasmic membrane. The release of the drug is thought to be catalyzed by protons that protonate the two Glu14, thus coupling drug export to H⁺ import. However, the precise mechanism by which the proton-induced conformational changes bring about translocation of substrate across the membrane remains to be deciphered. Table 2 lists some examples of SMR pumps and their substrates.

### 3. MATE pumps

MATE (Multidrug And Toxic compound Extrusion) MDR pumps are a relatively new family of secondary efflux pumps (67). Early members, NorM and its *E. coli* homologue YdhG were originally classified as MFS pumps because they possessed 12 putative transmembrane domains (68). However, it was later discovered that there was little sequence homology of these pumps with other MDR efflux pumps and they were reclassified as members of a new family of MDR pumps (MATE) (8). Since that time over 1000 members of this family, including representatives from all three kingdoms (Eukarya, Archaea and Eubacteria) have been identified (8, 19) and placed into three large subfamilies: 1) bacterial; 2) eukaryotic; and 3) bacterial and archaeabacterial transporters. MATE proteins range in size from 400-700 amino acid residues with 12 putative transmembrane α-helices. No apparent consensus sequence is found in all MATE sequences but they do share approximately 40% sequence similarity (67).
Bacterial MATE MDR pumps are energized by either H+ or Na+ coupled antiporters (68-70). These secondary MDR pumps can remove cationic drugs such as ethidium bromide, tetraphenylphosphonium, acriflavine, norfloxacin and berberine and at least one, MepA of S. aureus, when over expressed, can confer resistance to tigecycline, a new glycylcline antibiotic that is effective against methicillin resistant S. aureus cells (MRSA) (71). Because of their recent discovery, MATE MDR efflux pumps are the least well characterized and relatively little is known about their structure-function relationship. Most studies have focused on describing their presence and the antibiotic resistance they provide. However, with the recent crystallization of NorM from Neisseria gonorrhoeae (72, 73) this may change. Table 3 provides some examples of MATE MDR efflux pumps and their substrates.

4. MFS Pumps

The MFS (Major Facilitator Superfamily) pumps are proton-dependent secondary transporters. Approximately 25% of all known membrane transport proteins in bacteria belong to this superfamily (20, 85), which contains over 50 distinct families and more than 1500 members (15, 17, 86, 87).

Structurally, most MFS transporters are 400-600 amino acid residues in length and contain 12 or 14 TMDs (17), although there are at least two exceptions: one family has only 6 TMD and another 24 (15). There is also good evidence for an internal tandem gene duplication indicating a common ancestral gene. The best characterized MDR MFS pump protein is EmrD, an efflux pump that exports amphipathic compounds, such as CCCP, across the E. coli cytoplasmic membrane. Its crystal structure has been solved to 3.5 Angstrom resolution (88). It is 394 amino acids in length and has 12 TMDs organized as a pair of six-helix domains that surrounds a hydrophobic pore. Two long loops extend into the inner leaflet of the cytoplasmic membrane that are thought to determine substrate specificity, called a “substrate specificity filter”, and which may facilitate transport (88). It has been postulated that the mechanism by which MFS transporters move substrates across a membrane is via a “Rocker Switch Mechanism Alternating-Access”
model coupled with $H^+$ antiport (Figure 4). This model was originally based on the crystal structures of GlpT (89), LacY (90), and more recently EmrD (88).

Some multidrug resistant MFS systems have a tripartite structure (as do some ABC and RND pumps) such as the VceABC pump of *Vibrio cholerae* (91, 92) and the EmrAB-TolC pump of *E. coli* (93, 94). These pumps are responsible for the removal of substrates across both membranes of the Gram-negative bacterial envelope. These systems are comprised of a MFS transporter containing 14 TMD (EmrB, VceB), a periplasmic MFP (EmrA, VceA) and an OEP (TolC, VceC). Interestingly, unlike the tripartite AcrAB-TolC pump, where AcrB and TolC have been shown to be trimers, electron microscopy studies of reconstructed EmrAB suggest that they exist as dimers (94). More structural studies are warranted to decipher the interactions and architecture of MDR MFS tripartite efflux pumps. Table 4 presents some examples of MFS MDR efflux pumps and their substrates.

5. RND Pumps

RND superfamily transporters are found in Eukarya, Archaea and Eubacteria and have been placed into eight phylogenetically distinct families that correlate with their substrate specificity (123). RND transporters are larger than MFS transporters and range in size from 700 to over 1300 amino acid residues in length (124). Like MFS transporters, RND transporters are predicted to adopt a 12 TMD structure and the sequences of the first half and second half of the RND transporter are similar, suggesting that they also have arisen through gene duplication (124). However, unlike MFS transporters, RND transporters possess large periplasmic domains (34, 125, 126).

The crystal structure of the RND transporter, AcrB, has been solved (125, 127-129). It is a homotrimer, the monomer of which is 1049 amino acids in length, contains 12 transmembrane $\alpha$-helices, and two expansive periplasmic loops that determine substrate specificity (126, 130). Topologically, the core of AcrB is formed by a bundle of the 12 transmembrane $\alpha$-helices two of which (TM4 and TM10) extend approximately 70 Angstroms into the periplasm forming a distal
"TolC docking" domain and a "porter/pore domain" the latter being closest to the plane of the outer leaflet of the cytoplasmic membrane. In the center of the trimer, the TolC docking domain produces a funnel-shaped structure with a narrow diameter that leads to a central pore that is located in the porter domain. It is this domain with which AcrB docks to TolC. The central pore leads to a central cavity approximately 35 Angstroms in width. Three vestibules located near the cytoplasmic membrane have been implicated as entrances by which substrates may gain access to the central cavity (129).

The initial structural studies were conducted on crystals with three-fold symmetry. However, recently, reports describing an asymmetric AcrB trimer have been published (131, 132). The asymmetric structure reveals three different monomer conformations, presumably representing three consecutive states in the transport cycle and suggest a model for drug transport based on conformational cycling of the monomers by the RND pump (131-134). The three different monomer conformations are designated as loose (L), tight (T) and open (O) (131, 132, 135, 136). In this model, conformational changes from loose to tight to open and then back to loose enable the substrate access to a tunnel through which substrates are translocated to the outside via the TolC channel. The mechanism by which this is accomplished is based on occlusion migrating from the entrance toward the central tunnel similar to that of a peristaltic pump. The energy for the conformational cycling is envisioned to be provided by electron motive force (emf) to this transporter (See reference 135 for an excellent review of this mechanism).

6. Tripartite pumps

RND, MFS and ABC transporters can form tripartite pumps in Gram-negative bacteria. The components of these tripartite MDR pumps, as previously described, are periplasmic MFPs, outer membrane OEPs and the respective transporter protein (RND/MFS/ABC). All three components are essential for their function. The composite structure spans the cytoplasmic membrane, the periplasmic space and the outer membrane allowing for the removal of the substrate from the cytosol/ cytoplasmic membrane to outside of the cell envelope (Fig. 5). This provides a huge advantage for the Gram-negative bacterial cell because once exported, the drug must negotiate
the outer membrane barrier to reenter the cell. Thus as was so insightfully pointed out by Nikaido, these MDR pumps work synergistically with the outer membrane barrier (137, 138). That both the outer membrane barrier and MDR efflux pumps play an important role in the intrinsic resistance to various hydrophobic inhibitors was shown by the additive effect of deep rough (effects outer membrane permeability to hydrophobic agents) and MDR efflux (TolC) mutants (139).

The most extensively studied tripartite pumps are the MexA/MexB/OprM pump of *Pseudomonas aeruginosa* and the AcrA/AcrB/TolC pump of *E. coli*, both of which are considered to play a major role in antibiotic resistance for their respective bacteria. The RND transporters, MexB/AcrB determine the substrate specificity of their tripartite pump, which is quite large compared to other MDR pumps and combined include bile salts, organic solvents, dyes, and compounds that are anionic, cationic, zwitterionic, and a broad range of different antibiotics (133) (Table 5).

i. MFPs

Membrane fusion proteins are associated with their cytoplasmic membrane as either a lipoprotein or via a TMD near the N-terminal with the preponderance of the protein residing in the periplasm (181). Partial crystal structures of membrane fusion proteins MexA (140), AcrA (141) and MacA (142) are available (i.e. missing their extreme N- and C-terminal regions). The structures of AcrA and MexA share a significant degree of sequence and structure similarity. Both are elongated, sickle-shaped molecules composed of three domains: a β-barrel domain, a centrally located lipoyl domain and a coiled-coil α-helical hairpin at the other end of the molecule. Chemical cross-linkage and mutagenesis studies have shown that the α-helical coiled coil hairpin of AcrA/MexA docks with the coiled-coils of the OEP (TolC/OprM) (143, 144). The β-barrel domain is the probable site of interaction with the transporter protein (AcrB/MexB). The stoichiometry and oligomeric state of the assembled MFPs are unknown (See reference (145), for an excellent review on this topic).

ii. OEPs
The architecture of the OEPs whose crystal structures have been solved (*E. coli* TolC; *P. aeruginosa* OprM; and *Vibrio cholerae* VceC) are remarkably similar, even though their amino acid sequence identity/similarity is quite low (146-148). In each case the homotrimers of these proteins make up a long “cannon-shaped” structure consisting of a 40-Ångstrom-long β-barrel, which passes through the outer membrane and a 100-Ångstrom-long α-helical barrel which projects into the periplasm, which is closed at its periplasmic end (146). Based on this structure and the crystal structures of AcrB (129) and AcrA (141) and with the evidence that TolC could be cross-linked independently to either AcrA or AcrB (149-152), models have been proposed which attempt to explain the assembly and function of MDR pumps (143, 152-158). In such models, the periplasmic ends of a trimeric AcrB and trimeric TolC are envisioned to dock in such a manner as to form a continuous channel that crosses the periplasm and spans the outer membrane. The periplasmic contact between AcrB and TolC has been suggested to involve the TolC entrance coils and the apex (TolC docking domain) of AcrB (129, 154). In these models this connection is bridged and stabilized by the MFP, which is anchored to the cytoplasmic membrane and may play a role in the recruitment of TolC to the AcrB antiporter (159). During the assembly of the MDR pump, the periplasmic end of the OEP must open in order for the pump to function. This transition to the open state has been likened to an “iris-like” realignment of the entrance helices (146, 160). This opening of TolC is thought to occur through conformational changes in TolC via its interaction(s) with either AcrB or AcrA or both (146). However, the details by which tripartite pumps are assembled is only beginning to be deciphered and is currently an active area of investigation (see references (138, 155) for excellent reviews on this topic).

There are several different mechanisms by which an organism can become resistant to antimicrobial drugs. However, resistance mediated by multidrug resistance (MDR) efflux pumps appears to be a dominant paradigm among microbial human pathogens. Mobile genetic elements such as plasmids and transposons carrying genes encoding MDR pumps are thought to play an important role in the lateral acquisition of drug-resistance by bacteria. The emergence and increasing numbers of drug-resistant pathogenic bacteria pose a great threat to human health. Therefore, it is imperative to study the origin, evolution, and organismal distribution of
these xenobiotic transporters, especially in order to develop effective strategies to combat human diseases.

B. Phylogeny and Evolution of Bacterial Efflux Pumps

As noted above, one-tenth of the transporters encoded in a bacterial genome are involved in MDR efflux. The transporter classification system (14) currently recognizes approximately 650 transporter families that include about a dozen large superfamilies (http://www.tcdb.org/). A majority of these families are associated with solute uptake and only a few constitute exporters. Furthermore, only half-dozen exporter families (that include four of the superfamilies) contain members that extrude xenobiotic compounds. In spite of the fewer proportions of these xenobiotic-extruding transporters in organisms and their relatively high importance, these classes of exporters remain less well understood compared to the uptake systems (13).

The capability to export xenobiotic drugs appears to have evolved across independent lineages of transporters. As described above, thus far, functionally characterized bacterial drug exporters and their sequence homologs identified from genome analyses belong to one of five phylogenetically distinct and ubiquitously-found transporter families— the MFS superfamily, the RND superfamily, the DMT superfamily, the MATE family, and the ABC superfamily (13). It should be noted that not all families within each of these superfamilies are involved in drug efflux, and that many carry out solute uptake or export. It is likely that the drug efflux pumps function to export cellular metabolites and other molecules, and perhaps simple modifications in their protein sequence can confer additional capabilities to export either specific or multiple xenobiotics.

The MFS superfamily comprises of approximately 65 families of transporters of which only six contain characterized drug efflux pumps (Table 6). Similarly, only eight families within the ABC, two families within the RND, and only one family in the DMT superfamily (i.e., the SMR family) function in drug efflux in bacteria (Table 6).

II. Genomics of Bacterial Efflux Pumps

Since the first complete genome sequence of *Haemophilus influenzae* that was completed by
The Institute for Genomic Research (TIGR), hundreds of bacterial genomes have now been sequenced. Data from these genome sequences are constantly revealing many new uncharacterized transport proteins. In comparison to the few multidrug efflux transporters that have been functionally characterized, the genome sequencing efforts have identified numerous putative xenobiotic efflux pumps. Previous analyses of complete genomes indicate that approximately 10% of the transporters encoded in bacterial genomes are involved in multidrug efflux (3).

Comparative genomic analyses offer comprehensive overview of the distribution of transporters across a wide group of organisms. Such analyses offer distinct advantages that can help answer many biological questions. For example, 1) Are xenobiotic efflux pumps encoded in all bacterial genomes or occur only in certain species? 2) Is the distribution of the efflux types uniform or species-specific? 3) Did specific efflux transporters arise specifically in response to the use of drugs? This section of the review will focus on answering some of these questions.

**A. Methodology**

Sequences of all the predicted proteins in the complete genome sequences (which include chromosomes and plasmids) of 854 bacteria were downloaded onto our local computer from the National Center for Biotechnology Information (NCBI) through their ftp site (ftp.ncbi.nih.gov). A total number of 3,092,197 proteins sequences were obtained. The Basic Local Alignment Search Tool (BLAST) was also downloaded from the NCBI ftp site. All transporter proteins present in the Transporter Classification Database (TCDB; (184) were also downloaded onto our local machine. A total number of 5,238 functionally described transport proteins were obtained from TCDB. Of these, 236 transport proteins belong in the drug efflux transporter families described in Table 6. All 3,092,197 predicted proteins were searched against the 5,238 known transport proteins using the BLAST tool on our local machine. Expect value (E-value) cut-off of $10^{-6}$ was used for the BLAST search, this cut-off has been found from our previous studies to yield true hits and minimized false positives (185, 186). Proteins that showed one of the 236 MDR proteins in TCDB as their top most BLAST hit were identified and carefully inspected for any false positives.
A total number of 30,564 putative MDR efflux transport proteins from all the five major MDR efflux transporter classes were identified from the 8454 bacterial genomes. The organismal and taxonomical distributions of these transporter proteins will be described below. It must be mentioned that few distant homologs of the MDR pumps that fall below our search threshold (i.e., false negatives) may have been missed. However, from our previous experiences, we anticipate that the numbers of such false negatives would be extremely low.

B. Distribution of Drug Efflux Pumps in Bacterial Genomes

The genome sizes of the bacteria analyzed ranged between 0.25-10 Mb, with the exception of a myxobacterium belonging to the deltaproteobacteria subdivision, *Sorangium cellulosum* 'So ce 56', which has 13 Mb genome that is 71% G+C rich, and encodes 9381 predicted proteins (187). Our computational analyses identified a total of sixty-four MDR proteins in its genome. The smallest genome (0.25 Mb) analyzed was that of *Candidatus Sulcia muelleri* GWSS, a gut symbiont of the Blue-Green Sharpshooter and several other leafhopper species (188). The genome of this organism is only 22% G+C rich and is predicted to encode just 227 proteins. We could not identify any xenobiotic efflux proteins in the genome of this organism, representing the only organism lacking any recognizable xenobiotic transporters. At least one MDR efflux transport protein was identified in the remaining 854 bacterial genomes.

Of the collection of 30,564 drug transport proteins identified in the 854 genomes, approximately 33% (10,013 proteins) belong to ABC superfamily, 31% (9,349 proteins) are MFS-type, 27% (8,235 proteins) RND-type, 6% (1,810 proteins) MATE-type, and 4% (1,157 proteins) are SMR-type. A majority of MFS- and MATE-type pumps are generally composed of a single membrane translocator, while RND-type transporters commonly consist of two membrane-associated proteins. Although several SMR pumps are known to contain a single protein, there are many examples of SMR pumps composed of more than one protein. The drug-transporting ABC pumps may be composed of single protein with both the membrane-spanning and ATP-hydrolyzing domains fused, or may comprise of two separate proteins containing the two individual domains. Therefore, based on the adjusted calculations, we estimate that the relative abundance of the
different drug efflux pumps would be MFS (41%) > ABC (29%) > RND (18%) > MATE (8%) > SMR (4%). This shows that the MFS and ABC-type pumps are abundantly present in bacteria, while the MATE and SMR families have restricted representation. All five types were found to occur in a wide variety of bacteria and are not restricted to pathogenic bacteria, as noted above.

C. Correlation with Genome Size

In general, the total number of proteins dedicated to xenobiotic efflux in the bacterial genomes correlated ($R^2=0.72$, $p<0.05$) with their genome size (Fig. 6). The slope of the best-fit line was 10.36 (Fig. 6) indicating that approximately 10 drug transport proteins are encoded per Mb of bacteria genome. This is in good agreement with previous observations that 10% of all transport proteins in prokaryotes are involved in multidrug efflux (3). In general, a megabase of genome in bacteria encodes roughly 1000 proteins, and approximately 10-15% of all predicted proteins in bacterial genomes are transport proteins (189). Therefore, one would expect to find about 100-150 transport proteins encoded per Mb of genome, of these 10-15 proteins would be involved in xenobiotic efflux. In our analyses, the organism encoding the highest number of drug efflux proteins was *Burkholderia multivorans* ATCC 17616, which has a genome size of 7 Mb (190); it encodes 158 xenobiotic efflux proteins. This number is more than double the number of MDR efflux proteins expected for its genome size. *B. multivorans* is associated with infections in cystic fibrosis patients and is an important opportunistic pathogen that colonizes the lungs. It is associated with a decrease in long-term survival of patients. A minority of patients with *B. multivorans* infection develop "cepacia syndrome", which is frequently fatal (191).

Figure 6 also shows the correlation between genome size and the numbers of each of the five types of xenobiotic efflux pumps. The data show a relative abundance of MFS and ABC proteins, followed by the RND homologs. The MATE and SMR type occur in substantially lower proportions in the genomes. There appears to be an expansion of MFS-type of transporters with genome size, and some of the larger genomes contain abundant MFS transporters. This is likely because most MFS pumps comprise of a single protein and a single gene duplication can give rise to a functional new pump. Such an expansion is less pronounced with ABC and RND type pumps,
probably because of the multicomponent nature of these systems. Evolution of a new functional pump would involve coordinated duplication of multiple genes, which perhaps occurs less frequently. *B. multivorans* ATCC 17616 encodes the highest number of MFS and RND proteins (66 and 48 proteins, respectively) among all bacteria compared in our study, this is reflective of the large complement of drug transporters in this opportunistic pathogen. However, *Streptomyces griseus* subsp. griseus NBRC 13350, with slightly larger (8.5 Mb), encodes the largest number of ABC transport proteins (58 homologs) It is followed by seven other actinobacteria, two of which contain smaller genomes. *Corynebacterium glutamicum* ATCC 13032 with a genome size of 3.3 Mb, and *Beutenbergia cavernae* DSM 12333 with a genome size of 4.7 Mb, both encode 44 ABC proteins each. This indicates a relative expansion and likely importance of ABC family drug transporters in actinomycetes, soil-dwelling microbes that are known to produce a wide range of antibiotics. Both SMR and MATE transporters do not appear to increase in numbers linearly with genome size. Most organisms appear to encode 2-4 MATE-type and 1-2 SMR-type drug efflux transporters. *Bacillus licheniformis* ATCC 14580 encodes an expanded repertoire of 12 SMR family proteins. This organism is a soil-dwelling endospore-forming microbe that is used extensively in the industrial production of important enzymes such as proteases, penicillinases, and amylases as well as smaller compounds like the antibiotic bacitracin and various organic metabolites. Its 4.2 Mb genome encodes 125 drug efflux transporter proteins (3 times the expected number) representing all five families. The organism with the largest number of MATE family proteins is *Eubacterium eligens* ATCC 27750, a Firmicute and member of the normal human gut microflora. This organism (with 2.8 Mb genome) encodes a total of 46 drug efflux proteins that include the 23 ABC, 21 MATE and 2 RND family proteins but no MFS or SMR pumps. Thus, expansion of the MATE transporters may compensate for the lack of MFS facilitators in this organism.

**D. Organisms Lacking One or More Transporter Types**

Although just one organism was found to lack any recognizable drug efflux pumps as mentioned above, less than half (47%) of the bacteria analyzed contain all five types of drug transport
proteins (Table 7). This group of bacteria contains representatives from most of the major taxonomic subdivisions of eubacteria. Fifty-three percent of bacteria (455 genomes) were found to lack one or more of the five transporter types (Table 7). Approximately, 5% of bacteria (39 genomes) carry just one type of drug efflux transporters; many of these are intracellular pathogens and obligate symbionts. The genome sizes of these organisms ranged from 0.42 Mb to 1.9 Mb; all except one (Baumannia cicadellinicola str. Hc [Homalodisca coagulata]) lacked the secondary carriers, i.e., MFS, RND, SMR, and MATE porters, and contained 1-10 proteins of just the ABC-type transporters. These organisms include 23 mycoplasmas and 13 chlamydaiae that are known to have undergone genome reduction. A reduced genome (0.42 Mb) gammaproteobacterium, Buchnera aphidicola str. Cc (Cinara cedri), also contains just 2 ABC-type drug transport proteins, however, other B. aphidicola strains additionally contain MFS-type transporters. A Bacteroidetes with 1.9 Mb genome, Candidatus Amoebophilus asiaticus 5a2, encodes 10 ABC-type drug efflux proteins, but none of the other types. This organism is an obligate endosymbiont specific to its protozoan host, Acanthamoeba sp. TUMSJ-321, isolated from lake sediment in Malaysia (192). B. cicadellinicola str. Hc, a gammaproteobacterium, has a 0.69 Mb genome (193) and encodes just three MFS-type drug efflux proteins; it lacks any recognizable drug transporters of the other four types. This newly discovered organism is an obligate endosymbiont of the leafhopper insect Homalodisca coagulata (Say), also known as the Glassy-Winged Sharpshooter, which feeds on the xylem of plants.

Forty-one (5%) organisms encode only two out of the five efflux types, many of which also are intracellular pathogens and endosymbionts. Of these, twenty-six contain just MFS and ABC-type drug efflux transporters, seven encode MFS and RND type pumps, four encode ABC and RND type pumps, three contain ABC and MATE type transporters, and only one organism encodes RND and MATE family of transporters but not the other types. The organisms containing both and MFS and ABC type transporters comprise predominantly of Firmicutes (17 species, mostly Streptococcus pyogenes strains), Gammaproteobacteria (4 Buchneria aphidicola strains), Alphaproteobacteria (3), and Actinobacteria (2 Tropheryma whipplei strains). The remaining organisms that do not contain either MFS or ABC type efflux pumps are Alphaproteobacteria (7),
Spirochetes (2), Bacteroidetes (2), Gammaproteobacteria (2), a Firmicute, and an unclassified bacterium; most encode less than 10 drug transporter proteins. However, two organisms encoded as many as 19 and 30 efflux proteins each and are briefly discussed below. *Halorhodospira halophila* SL1, a Gammaproteobacterium, has a genome of 2.7 Mb genome and encodes 8 ABC-type drug transport proteins and 11 RND-type efflux transporter proteins. *H. halophila* SL1, formerly *Ectothiorhodospira halophila*, was isolated from salt lake mud and is one of the most halophilic eubacteria known (194). The other organism, *Eubacterium rectale* ATCC 33656, with a 3.4 Mb genome encodes equal number (fifteen each) of ABC and MATE-type drug transport proteins. *E. rectale* ATCC 33656, a Firmicute, was isolated from human feces. *Eubacterium spp.* are thought to play a beneficial role in maintaining the normal ecology of the large intestine, in part by producing chemicals like butyric acid which act to inhibit the growth of other bacteria. These organisms are occasionally isolated from wounds and abscesses and may be an opportunistic pathogen. This genus has also been isolated from sewage and soil.

One hundred and seventeen (14%) of the bacteria that contain three types of drug efflux transporters are predominated by bacteria (83 genomes) that contain MFS, RND, and ABC proteins (83 organisms) while twenty-one bacteria lack the MFS and SMR homologs, eleven lack the RND and SMR pumps, and two organisms lack RND and MATE transport proteins. The organisms that lack the MFS proteins comprise of Spirochaetes (8), Firmicutes (4), Gammaproteobacteria (4), Bacteroidetes/Chlorobi (2), Thermotogae (2), and Fusobacteria (1). Finally, 30% (258) of bacteria lack just one type of transporter, of which a majority lack SMR-type pumps (170 genomes), followed by MATE-type pumps (82 genomes). The MFS and RND pumps are absent in three genomes each, while there were no organisms that lacked just the ABC-type drug efflux transporters.

### E. Taxonomical Distribution of Drug Efflux Pumps

The bacteria surveyed in our present study can be classified into 19 different taxonomical groups. Gammaproteobacteria, Firmicutes, and Alphaproteobacteria are well represented with 221, 189, and 111 bacterial genomes, respectively. While Planctomycetes, Fusobacteria, and Acidobacteria
are poorly represented, having just 1-2 completely sequenced members, another thirteen
taxonomic subdivisions contain 5-69 bacterial species with complete genomes. The relatively
well-represented groups offer an opportunity to assess any taxonomic bias with respect to the
occurrence of drug efflux pumps. The average number of drug efflux transport proteins per
megabase of genome within each group of bacteria is shown in Figure 7. These data show that
both Firmicutes and Gammaproteobacteria contain the highest density of drug transport proteins
per Mb of genome, while the genomes of Chlamydiae/Verrucomicrobia contain four times lower
density of these proteins (Fig. 7). Spirochaetes and Cyanobacteria encode half as many drug
transporters as Firmicutes per unit length of the genome. Interestingly, the relative density of
each of the five transporter families varies remarkably across the bacterial groups (Fig. 7).
Firmicutes and Thermatogae encode more ABC proteins per Mb of genome, whereas
Betaproteobacteria, Epsilonproteobacteria, Spirochaetes, Chlamydiae/Verrucomicrobia, and
Aquificae encode only half as many of these transport proteins. Although Spirochaetes and
Chlamydiae/Verrucomicrobia encode fewer ABC transporters, the largest number of drug
transporters in these bacteria is the ABC-type, reflective of the overall lower abundance of drug
transporters in these bacteria.

Actinobacteria and Betaproteobacteria along with Firmicutes and Gammaproteobacteria encode
3-5 MFS proteins per Mb of genome, higher than most other groups. The highest number of drug
transporters in the Deinococcus-Thermus group is the MFS-type, and these bacteria encode 2-3
MFS transporters per Mb of genome. Bacteroidetes/Chlorobi as well as all Proteobacteria encode
more RND transporters per unit of genome, while Firmicutes have a much lower representation of
RND pumps in their genomes. MATE and SMR pumps generally occur in substantially lower
proportions in bacterial genomes. Interestingly, the single member of the Fusobacteria analyzed
encodes as many as 4 MATE family proteins per Mb of genome in comparison to the 0-2
homologs in other groups of bacteria. *Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25586
has 2.17 Mb genome and encodes 19 drug efflux transport proteins, of which, 8 are MATE-type,
6 are ABC proteins, and 5 are RND-family proteins. It appears to lack MFS- or SMR-type drug
transporters completely. *F. nucleatum* belongs to the normal microflora of the human oral and
gastrointestinal tracts (195). This bacterium is capable of forming coaggregates with other pathogenic and non-pathogenic bacteria in the mouth. Its MATE-type efflux transporters may play a role in resistance to antimicrobials produced by other inhabitants of the oral microflora.

**F. Differences among Strains.**

Approximately, half of the bacterial species analyzed (431 genomes) are represented by a single sequenced strain, while 111 different bacterial species have multiple strains (ranging between 2-27 strains) whose genomes have been completely sequenced, accounting for the remaining 423 genomes. Thirty-seven bacterial species with four or more completely sequenced strains will be discussed below (Table 8).

*Escherichia coli* has the most number of strains sequenced (27 strains); these include both pathogenic and non-pathogenic strains. Six other species contains ten or more sequenced strains, these are, *Salmonella enterica* (15 strains), *Staphylococcus aureus* (14 strains), *Streptococcus pyogenes* (13 strain), *Prochlorococcus marinus* (12 strains), *Streptococcus pneumoniae* (11 strains), *Clostridium botulinum* (10 strains). Surprisingly, the strain-level variation in the numbers of the different drug efflux pumps appears to be very low in *S. aureus* and *S. pyogenes*, both pathogenic in human.

Six sequenced strains of *Acinetobacter baumannii* show large variations in the numbers of drug transporters (Table 8). Most notable are the differences in the numbers of MFS transporters, followed by RND, SMR, and ABC proteins in the descending order. *A. baumannii* is an aerobic Gram-negative bacillus that is an opportunistic pathogen in humans. Infections by this organism are becoming increasingly problematic due to the high number of resistance genes found in clinical isolates. Some strains are now resistant to all known antibiotics. Most of these resistance genes appear to have been transferred horizontally from other organisms. Many of them cluster into a single genomic island in strain AYE as compared to strain SDF.

Similar high level of variation is observed in six strains of a non-pathogenic bacterium, *Rhodopseudomonas palustris*, which is commonly found in soil and water environments (Table
However, the most noted differences were observed in the numbers of ABC and RND-type of pumps, and to a lesser extent in the number of MFS drug transporters. Intermediate level of differences are found among strains of other pathogenic species such as *Bacillus anthracis*, *Bacillus cereus*, *Coxiella burnetii*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Yersinia pestis*, as well as non-pathogenic species such as *Pseudomonas putida*, *Shewanella* sp., and *Synechococcus* sp (Table 8). In the above six pathogens, the differences are mostly in the numbers of MFS and ABC transporters, except in the case of *C. burnetii* and *E. coli*, whose strains mostly differ in the complements of MFS and RND family of proteins. A few additional pathogens with interesting profiles are as follows: *S. enterica* strains differ in the number of SMR proteins besides MFS and RND; *C. botulinum* strains show differences in the number of MATE homologs in addition to ABC and MFS proteins; strains of *Francisella tularensis* and *Burkholderia cenocepacia* vary mostly in the number of predicted MFS-type drug efflux pumps, and *S. pneumoniae* strains mostly show differences in the number of ABC proteins (Table 8). Strains of all other species show relatively fewer differences in the number of drug efflux transporters encoded in their genome (Table 8).

The organization differences between prokaryotic (bacterial) and eukaryotic cells (animals, plants, fungi) in part impose requirements for transport proteins to move molecules into and out of cells. Gram-positive bacteria are enclosed by a single cytoplasmic membrane while Gram-negative bacteria possess two membranes; an inner cytoplasmic membrane and a lipopolysaccharide-containing outer membrane. Movement of molecules into and out of Gram-positive bacteria generally requires single-component transporters. Transport of molecules in Gram-negative bacteria requires passage through the periplasm enclosed between the two membranes. Thus many transporters in Gram-negative bacteria are multi-component as described in this chapter. The cytoplasm of fungi is enclosed by a single plasma membrane and therefore most transporters are products of a single gene (described below). However unlike Gram-positive bacteria, fungi also possess transport proteins in membranes of intracellular organelles. While the majority of fungal efflux pumps reside in the cytoplasmic membrane, some pumps are located in the membranes of vacuoles (196, 197).
III. Fungi

The fungi represent a large, diverse group of eukaryotic microorganisms that range in size from macroscopic, multicellular mushrooms to microscopic unicellular forms. Fungi inhabit most environments on the planet but their primary reservoir is the soil. While most may appear invisible many are essential for carbon and nutrient recycling in nature, others are important in the food, pharmaceutical and beverage industries and many are serious pathogens of plants and animals. Fungi are also known to synthesize a vast array of compounds, many of which are toxins. Fungi may form threadlike tubular walled structures, hyphae that branch and anastomose into complex mat-like structures known as mycelia. Hyphae may be segmented into individual cell-like units connected by pores that permit movement of organelles, nuclei and cytoplasm. Some fungi may exist as unicellular forms with cell walls and a single nucleus per cell. These yeast forms occur in many fungal groups. Dimorphic fungi possess both yeast and mycelial stages in their life histories and each growth form may provide novel functional capabilities to the organism. Thus plant- and animal-pathogenic fungi may utilize one developmental form during interaction with the host and the other during growth outside the host. Some fungi have no known mycelial stage and form sac-like cells which may be multinucleate (yeasts). Some of these fungi have motile flagellated forms as part of their life cycle and these often play a role in the initial interaction with the host. Almost all groups of fungi have members that are important pathogens of plants, animals and humans. Over the past two decades we have observed a sharp increase in the occurrence of invasive fungal infections of humans many of which are associated with morbidity and mortality. More recently, fungi have been implicated as the causative agents in the white-nose bat syndrome (Gleomyces sp) and in the global extinction crisis of amphibians (Batrachochytrium dendrobatidis).

A. Antifungal Agents and their Use

Because of their eukaryotic nature, fungi are inherently difficult to treat without causing damage to the host. Furthermore, their relatively slow growth rate (compared to bacteria), adds to the loss in efficacy of certain antifungal agents. These features, therefore, restrict the array of anti-fungal
drugs that can be used therapeutically (207). The limited number of useful drugs to treat fungal infections is under the additional burden because of rapidly developing antifungal drug resistance (207, 208). Thus the development of new drugs that impair uniquely fungal biological processes, with limited side effects and ease of delivery is of vital importance. Seven major classes of drugs are currently used to treat fungal infections therapeutically; the triazoles, polyenes, echinocandins, allylamines, nucleoside analogs, morpholines and griseofulvin-type. Among drugs currently used to treat fungal infections are those that target an enzyme in a unique fungal sterol biosynthesis pathway absent in plants and animals. The azoles (fluconazole, ketokonazole, itraconazole) are inhibitors of fungal P450_{14DM} (lanosterol 14α-demethylase) cause accumulation of C-14 methyl sterols in fungi and impair normal membrane function (209-214). The polyenes (amphotericin B and nystatin), intercalate into membranes containing the unique fungal sterol ergosterol causing ion leakage and cell death (215). Echinocandins (eg. caspofungin and micafungin), inhibit 1, 3-β-D-glucan synthase required for fungal cell wall synthesis. Loss of wall integrity can result in cell lysis and death. The azoles, polyenes and echinocandins are the only three of the seven antifungal drug classes that can be used to treat systemic infections (216). The allylamines (naftifine and terbinafine) inhibit squalene oxidase a key step in ergosterol biosynthesis (required for fungal plasma membranes). Morpholine (amorolfine) impairs ergosterol biosynthesis by inhibiting cytochrome P450 enzymes, nucleoside analogs, (flucytosine) and Griseofulvin-type (grifulvinV, fulvicin U/F) interfere with DNA and RNA synthesis and mitotic spindle formation, respectively (215). In nature, fungi encounter a large variety of antifungal substances that are made by a broad spectrum of organisms (217, 218). These compounds include peptides, fatty acids, proteins, alkaloids, quinones, and statins. Survival therefore necessitates employment of effective anti-toxin mechanisms. The most common processes used by fungi to become resistant to antifungal agents are destruction of the agent, change in the target enzyme or pathway by mutation and active efflux to maintain low intracellular concentrations (213, 219).

B. Fungal Efflux Transport and Genomics
Data from genome sequences available at the Broad Institute and The J. Craig Venter Institute (Transporter Protein Analysis Database) were used in this review (http://www.broadinstitute.org/; http://www.membranetransport.org/). A significant proportion of fungal genomes are devoted to transport proteins. Transport proteins are important for nutrient uptake, intracellular ion concentration maintenance, secretion of proteins, secretion of secondary metabolites, and efflux of toxins and xenobiotics. In the yeasts, *Saccharomyces* sp., and filamentous fungi, *Aspergillus* sp, *Neurospora* sp and *Cryptococcus* sp., the number of transport proteins per megabase of genome is between 13 and 30 (220). By comparison, the closely related fungal group, the Oomycetes have <5, and model eukaryotes *Arabidopsis thaliana* 9.7, *Caenorhabditis elegans* 6.7, *Drosophila melanogaster* 5, *Entamoeba histolytica* 8.5 and *Mus musculus* 0.4 (220). This underscores the importance of transport proteins to the survival of fungi in different environments. For example, in the soil, fungi have to contend with bacterial and fungal antibiotics, plant root exudates, chemicals from pollution, protozoa and insects. Pathogenic fungi have to survive in potentially toxic host plant or animal environments which necessitate effective efflux processes. Additionally, many fungi produce toxins and secondary metabolites that must be secreted into their hosts or the environment (221). Two major classes of transport proteins are found in fungi; the ATP-Binding Cassette Superfamily (ABC) and the Major Facilitator Superfamily (MFS) (222).

These two superfamilies of transporters make up between 12 to 22% and 76 to 85% respectively of the total number of transporters in many fungi. The ABC transporters belong to a large superfamily of membrane proteins that are found in other eukaryotes and bacteria. The nucleotide-binding domain (ATP binding cassette) is the most highly conserved region among members of this superfamily. The energy that drives movement of molecules across the membrane through these transporters is derived from ATP. Members of the superfamily are important in import of sugars, amino acids, peptides, ions and efflux of proteins, secondary metabolites and xenobiotics. All fungi examined thus far possess ABC superfamily transporters. Table 9 shows the proportion of fungal and related Oomycete, *Phytophthora infestans* efflux transporters out of the total number of transporters of each superfamily, ABC and MFS, from a distribution of pathogenic (P) and non-pathogenic (N) fungi. Greater than 50% and up to 75% of
the ABC transporters in these organisms are devoted to efflux purposes. While the number of *P. infestans* ABC transporters is large, they actually represent 0.67 per Mb genome and the number of ABC efflux transporters per Mb genome is 0.5. On the other hand the fungi *Aspergillus fumigatus*, *A. nidulans*, *C. neoformans* and *S. cerevisiae* encode approximately 1 ABC efflux transporter per Mb genome. *Neurospora crassa* however encodes just 0.4 ABC efflux transporters per Mb genome. These observations suggest that a sizeable portion of the transport protein-encoding genome in some fungi is committed to maintenance of an intracellular environment low in potentially harmful metabolites and xenobiotics or for secretion of toxins. Most of these data are based on bioinformatic evidence and more experimental evidence is required to support these observations. Interestingly there appears to be no correlation between the percentage of efflux transporters and whether a fungus is a pathogen or non-pathogen (Table 9). This suggests that efflux pumps play a variety of roles in addition to those required to survive in a toxic host environment. It is conceivable that resistance capabilities may have evolved from proteins required for other cellular and ecological processes. A similar concept has been previously discussed with fungi, where efflux pump gene expression is required during mitosis (223) and where important physiological substrates like steroids may be transported by efflux pumps (224, 225). Similar suggestions have also been made in bacteria where efflux pumps play roles in endurance in their ecological niches such as attachment, invasion, colonization and persistence (226).

1. Fungal ABC Transporters

ABC transporters may be organized into different configurations; transmembrane domains (TMDs) followed by nucleotide binding domains (NBDs), (TMD-NBD)$_2$, reverse (NBD-TMD)$_2$ or (NBD-TMD). Each nucleotide binding domain contains characteristic sequences [Gx4GK(ST)] Walker A box and [[(RK)X3GX3L(hydrophobic)] Walker B box separated by 90-120 amino acids (227). In fungi, full size transporters typically comprise 1200 amino acids, between 12 and 20 TMDs and 2 NBDs (TMD-NBD)$_2$ or (NBD-TMD)$_2$, whereas half-size transporters have between 5-10 TMDs and 1 NBD (NBD-TMD) (228). While the TMDs probably function in substrate
translocation across the membrane, the driving energy derived from ATP is harvested by the NBDs. The ABC superfamily of transporters comprises 7 families ABCA, ABCB, ABCC, ABCD, ABCE, ABCF and ABCG. Of these, families ABCB, ABCC and ABCG are implicated in active efflux and are also known as the multi-drug resistance (MDR), multi-drug resistance-associated protein (MRP) and the pleitropic drug resistance (PDR) families (229, 230, 231). Families ABCE and ABCF that do not have TMDs will not be discussed further (232, 233).

Saccharomyces cerevisiae is the best and most extensively studied model fungus. Consequently, we have much biochemical, physiological and molecular biological evidence to support the bioinformatic information on the organism. The S. cerevisiae genome encodes 24 ABC superfamily transporters and these represent approximately 34% of the ATP-dependant transporters (220). Representatives of the ABCG family (PDR5) are in a (NBD-TMD) 2 configuration and are involved in multidrug resistance. In this fungus, the family is also known as Cluster I and includes Pdr5p, Snq2p and YOl075C proteins (228, 234). S. cerevisiae ABCC family representatives (MRP; Cluster II.1) Ycg1p, Btp1p, Ybt1p/Bat1p and Yor1 have a (TMD-NBD) 2 configuration and are full-size transporters. The S. cerevisiae ABCB family representative (Cluster II.2) Ste6p is a full-size transporter. Cluster II.3 representatives of this family, Atm1p, Mdl1p and Mdl2p are half-size transporters.

i. Saccharomyces species

Substrates of efflux proteins Pdr5p, Snq2p and Yor1 were studied using single, double and triple mutants with 349 substrates (235). This study demonstrated that these pumps share overlapping though not identical substrate preferences. Triple mutants showed full sensitivity to itraconozole, miconozole, nystatin, antimycin and tetradecylammonium bromide. Pdr5p was observed to provide resistance to cycloheximide, benomyl and phenaprmyl, Snq2p to resazurin and quinoline oxides and Yor1p to propanil, ferbam, oligomycin and thiram. In an elegant study using point mutations with Pdr5p, it was demonstrated that substrate specificity is in part determined by protein folding and that pump inhibitor sites are functionally separated from substrate interacting sites (235). Pdr5p has also been shown to mediate resistance to certain mycotoxins, and is
involved in the transport of glucocorticoids (236, 237). ABCC family proteins of Cluster II.1 Ycg1p, Btp1p and Ybt1p/Bat1p are capable of transporting bile acids and glutathione conjugates. ABCB (Cluster II.2) family protein Ste6p is required for transport of mating pheromone (factor α). Cluster II.3 proteins are all localized to the mitochondrial membranes and play a role in export of mitochondrial peptides. While most of the S. cerevisiae ABC efflux transporters are localized to the plasma membrane (eg., Pdr5p, Snq2, Yor1p, Pdr10p, Pdr15p, Ste6p), others may be localized to vacuolar membranes (eg., Btp1p, Ybt1p, Ycf1p) (230).

ii. Aspergillus species

The genus Aspergillus contains members that have considerable impact on our lives and environment. Aspergillus fumigatus is an opportunistic pathogen of animals, while A. flavus infects grain crops and is responsible for the production of aflatoxin. Aspergillus nidulans is generally non-pathogenic and is a soil-borne fungus. Aspergillus fumigatus persists in the environment as airborne spores and consequently the human respiratory tract is constantly exposed to the fungus. Infections of immunocompromised individuals by A. fumigatus are very high and the mortality rate is as high as 50%. Recent observations of increased A. fumigatus resistance to triazoles have partly been attributed to enhanced drug efflux pump activity (238), (239). This fungus encodes 45 ABC transporters of which 75% are involved in efflux. Of these, 12 pumps belong to the ABCG family, 13 to the ABCB family and 10 to the ABCC family. BLAST pair-wise alignment shows that 12 efflux transporters in A. fumigatus, 2 transporters from A. nidulans and 10 transporters from A. flavus are related to the prototypical mammalian P-glycoprotein (ABCB family). A. flavus has 23 transporters that belong to the ABCG family based on pair-wise alignment while A. nidulans has 14 representatives. ABC proteins AfuMdr1 (A. fumigatus) and A. flavus AfIMdr1 are similar to the Schizosaccharomyces pombe leptomycin B resistance protein and also to the human Mdr1. A. fumigatus, AfuMdr2 is similar to two MDR-like genes of S. cerevisiae and confer resistance to the echinocandin B analog, cilofungin (240).

The genome of the non-pathogen A. nidulans encodes 45 ABC transporters of which 75% are involved in efflux. In A. nidulans, ABC superfamily efflux transporters bearing resemblance to the
S. cerevisiae Pdr5p and Snq2p transporters have been described experimentally (241). The proteins AtrA and AtrB also share homology to the dimorphic fungus Candida albicans, Cdr1 and mammalian P-glycoprotein-type transporters (see below). AtrB expression in S. cerevisiae was capable of countering drug hypersensitivity of a S. cerevisiae Pdr5p mutant. Interestingly, transcription of these A. nidulans transporters was found to be enhanced following exposure of the fungus to azoles or plant defense chemicals. A. nidulans AtrA, B, C and D genes were differentially expressed when the fungus was grown in the presence of structurally unrelated compounds camptothecin, imazalil, itraconazole, hygromycin, and 4-nitroquinoline oxide (4-NQO) (242). In the presence of 4-NQO, AtrA expression was increased approximately 14-fold but AtrB was increased more than 4500-fold. AtrC expression was enhanced more than 62-fold in the presence of hygromycin, and AtrD expression was enhanced more than 250-fold. Itraconazole in the growth medium enhanced AtrB and AtrD expression 39- and 23-fold respectively while exposure to camptothecin resulted in decreased expression of AtrA and AtrC. AtrA was also repressed in the presence of imazalil and itraconazole. These observations suggest that resistance to certain compounds may be preferentially provided by certain transporters. Alternatively, induction and repression of transport protein-encoding genes may respond differently to varying stimuli.

iii. Candida species

Candida albicans is a dimorphic fungus capable of causing opportunistic systemic infections in immunocompromised individuals and superficial mucosal infections in healthy individuals (243). Many of the systemic infections often result in mortality. In fact 50% of nosocomially acquired fungal infections are caused by this fungus (244, 245). Dimorphism in this fungus is characterized by a yeast-like budding form and a hyphal form that can develop into a mycelium. The hyphal-mycelial stage is often recognized with the onset of pathogenesis. In C. albicans, 13 proteins are identified as resembling the ABCG family (Pdr5p-like), 2 proteins bear significant identity to the ABCB family (-factor export), and 2 proteins show significant identity to the ABCC family (oligomycin resistance). In C. albicans Cdr1p (ABCG) provides resistance to azoles and in
certain clinical isolates has been shown to be important in resistance to fluconazole, ketoconazole and itraconazole (246, 247). Indeed over expression of the transporter is believed to play a significant role inazole resistance in clinical isolates of the fungus (248). Using photoaffinity labels in competition studies, Cdr1p has been shown to possess separate substrate binding sites for nystatin and myconazole (249). Site-directed mutagenesis of a conserved cysteine residue in the Walker A motif of Cdr1p suggests that the nucleotide binding domains respond asymmetrically to substitutions in this amino acid (250, 251, 252). Structure/function studies with this protein suggest that specific amino acid residues in the NBD are either indispensable or are important determinants in substrate affinity interactions (252). Furthermore, conformational changes in Cdr1p are unaffected by specific amino acid changes in Cdr1p but are impaired in others (252). In an elegant experiment, Cdr1p was functionally reconstituted into sealed membrane vesicles and shown to carry out drug efflux and also translocate phospholipids (253). Energy-dependant efflux using rhodamine, a fluorescent dye has been measured in Candida species (254).

While the ABC efflux transporters of C. albicans provide resistance to azoles, they have not been implicated in resistance to the cell wall inhibiting echinocandins (255). Interestingly, in C. dublinienisis however, the role of Cdr1p in fluconazole resistance has been questioned (256). This suggests that utilization of certain efflux pumps by different species of a fungal genus may vary and may provide clues to alternate mechanisms or pumps employed for drug resistance.

Compared to the animal pathogenic fungi biochemical and molecular biological experimental data in the plant pathogens are lacking. However, many fungal plant pathogen genomes have been sequenced and provide useful information. Plant pathogen efflux transporters have been recently reviewed (229).

Two broad categories of these pumps have been designated: those involved in secretion of virulence factors and toxins (mycotoxins eg. aflatoxin, gliotoxin and host-specific toxins eg, vitorin, botrydil and cercosporin) and those involved in removal of plant-derived antifungal agents. The efflux transporters of the first class will not be described further in this chapter. Fungal plant
pathogens have been shown to gain resistance to fungicides when exposed to low levels of the anti-fungal agents. Strobilurin is a “quinine outside inhibiting” (QoI) fungicide and is particularly useful against two different classes of fungi the ascomycetes and the basidiomycetes as well the related group, the oomycetes. The target of QoI fungicides is the mitochondrion and fungi have evolved different mechanisms to provide resistance to these compounds (257).

In *Pyrenophora tritici-repentis*, exposure of the fungus to sub-lethal concentrations of QoI fungicides showed increased efflux-based resistance to strobilurin and azole fungicides (258). Analysis of the *P. tritici-repentis* genome shows that it encodes 15 ABC transporters that belong to the ABCG subfamily, 47 ABCB subfamily proteins, and 38 ABCC proteins (E <10^{-12}). *Puccinia graminis-tritici* a pathogen of wheat, barley and oats (259) encodes approximately 10 ABCG subfamily proteins, 15 ABCB proteins and 15 ABCC subfamily transporters. The plant pathogen *Magnaporthe grisea*, also shows enhanced ABC efflux transporter gene expression following low level exposure to antifungal agents (260). This ABC efflux transporter has been shown to be an important pathogenicity factor during infection of rice (260). Gene expression of ABC1 was enhanced following exposure to toxins and a rice antifungal phytoalexin, sakuranetin. Interestingly, a mutation in the gene did not result in hypersensitivity to antifungal agents tested (inhibitors of protein synthesis, sterol biosynthesis, protein secretion and a phytoalexin). The mutant was however unable to cause disease in rice plant bioassays. These results suggest that in vitro susceptibility testing may not always provide an accurate perspective of the role of a protein or proteins during infection and/or that the MgAbc1 protein may play additional roles during the infection process (for example secretion of toxins or efflux of compounds not tested in vitro). The ABC3 transporter in *M. grisea* has been shown through mutational analysis to provide fungal resistance to peroxide and other cytotoxic agents of plant origin during the early stages of infection (261). Analysis of the genome of *M. grisea* indicates that it encodes approximately 15 proteins of the ABCG subfamily, 66 proteins of the ABCB subfamily and 52 of the ABCC subfamily (E <10^{-12}). The genus *Fusarium* causes disease on over 200 species of plants, many of which are of agricultural importance. Among these are legumes, cereals and wheat. Three genera *F. oxysporum*, *F. graminearium* and *F. verticilloides* encode 50, 36 and 31 proteins that
belong to the ABCG subfamily, 56, 57 and 55 of the ABCB family and 56, 57, and 55 ABCC proteins respectively, signifying the importance of these efflux transporters to these pathogens. Recent documentation of increased benzimidazole resistance in *F. graminearum* in China (262) may be due to enhanced activity of these transporters. *Verticillium dalliae* and *V. albo-atrum* pathogens of woody plants (eg., ash, elm, oak, maple) encode 22 and 27 ABCG proteins, 49 and 48 ABCB transporters and 34 and 35 ABCC transporters respectively. *Ustilago maydis* a basidiomycete pathogen on grain crops encodes 13 ABCG proteins, 44 ABCB transporters (E <10^{-12}). *Mycospharella graminicola* a pathogen of wheat has been experimentally shown to have resistance to azoles conferred by ABC transporters (263). *Botrytis cinerea*, a pathogen of grapes, ornamentals, fruit and vegetables encodes approximately 21 ABCG subfamily proteins, 47 ABCB proteins and 47 ABCC transporters (E <10^{-12}). Reduced susceptibility of this pathogen to the fungicides fludixonil and fenpiclonil is moderated by the ABC efflux transporter, BcatrB (264). A mutant with a disruption in the BcATRB gene showed higher accumulation of fludixonil and reduced accumulation in strains in which the gene was over-expressed. The use of azoles to control plant pathogenic fungi has been considered from the perspective of enhancing resistance to these compounds in animal pathogenic fungi (233, 265). While the debate is far from over, it is apparent that use of antimicrobial agents in agriculture may result in them leaching into water systems and soils thereby exposing other pathogens to sub-lethal concentrations that may result in increased resistance broadly.

The amphibian pathogen *Batrachochytrium dendrobatidis* a chytrid fungus has been implicated in the global decline of frog populations (266). This pathogen infects the keratinized mouth parts of tadpoles and keratinized epithelial cells of adults. Little is known of how the fungus infects it host, survives within the host epithelial cells or survives in the environment. Analysis of the genome sequence shows the presence of 17 ABCG efflux transporters, 64 ABCB transporters and 33 ABCB subfamily transporters (E <10^{-12}). No experimental data are available on the role of these efflux transporters in the survival and infective stages of the pathogen.

**iv. Phylogenetic Analysis of Fungal ABC Transporters**
Because evolutionary converge of function implies convergence of DNA or protein sequences, a phylogenetic perspective can be used to assess whether pathogenic fungal species are more similar in sequence than is expected from their phylogenetic relationships. Several methods are available for evaluating converge within a phylogenetic context (267, 268, 269). One such method is illustrated here, based on a phylogenetic hypothesis (Figure 8) of the relationships among a set of 27 species of ascomycota fungi based on amino-acid sequences of the Candida ABC efflux transporter (Cdr1p), which has been characterized biochemically. About half of the species (14 of 27, indicated by ‘P’ following the strain identifier) are pathogenic; the remaining 13 species (indicated by ‘N’) are non-pathogens. To the extent that the pathogens are convergent in protein sequence, they should tend to cluster together on the tree; if their sequences are not convergent, then the tree should instead reflect their evolutionary relationships. There is no obvious clustering of pathogenic and non-pathogenic species, independent of taxonomic relationships. The basal branch of the tree (estimated by mid-point rooting) divides the species into the classes Saccharomycetales and Eurotiomycetidae (Ascomycetes). Although some of the relationships among genera are unexpected (e.g., the inclusion of Coccidioides, Penicillium and Neosartorya within Aspergillus), such lack of resolution is common in single-gene analyses.

Ideally the tree should be compared against a comparable phylogenetic tree, for the same species, based on multigene or phylogenomic analyses (270, 271, 272; 273, 274). Because such a phylogenetic study has yet to be done, the tree of Figure 8 can more crudely be compared with a classification based on the Linnean hierarchy (derived from The Global Biodiversity Information Facility; //www.gbif.net/species/browse/taxon/13140889), using the method of Podani (275) to detect hierarchical levels at which the two dendrograms show maximum agreement. The resulting comparison indicated strong similarities at all levels in the trees, with marginally significant disagreement only in the dispersion of Aspergillus species. Thus the phylogenetic tree based on Cdr1p sequences is strongly consistent with the accepted Linnean classification, with no evidence supporting either sequence convergence or pathogenic patterns conserved among species (274).
2. Fungal MFS Transporters

The Major Facilitator Superfamily (MFS) proteins in the fungi are relatively poorly understood when compared to the ABC transport proteins. A recent review of fungal MFS proteins and their roles in fungal physiology underscores this point (281). Major Facilitator Superfamily efflux transporters possess either 12 (Drug: Proton Antiporter, DHA1) or 14 (DHA2) TMDs with a large cytoplasmic loop between domains 6 and 7. Analysis of the *S. cerevisiae* genome sequence indicates the presence of approximately 85 MFS transporters of which 21% are involved in efflux (Table 9). Only 3 of these proteins have been functionally characterized. Among other fungi the percentages of MFS-MDR transporters range between 29 and 39% (Table 9). Curiously in the fungal related group, the Oomycetes, (*P. infestans*) less than 2% of the MFS proteins are involved in efflux. This is particularly interesting because the percentage of ABC efflux transporters in *P. infestans* is not notably different from the other fungi.

One of the first MFS proteins involved in drug resistance in fungi was identified in *S. cerevisiae* through mutant selection and complementation experiments (282). The MFS efflux transporter Dtr1p known to provide resistance to organic acids, and antimalarial drugs has also been shown to play an essential role in *S. cerevisiae* spore wall maturation by secreting the building black, bisformyl tyrosine from the cytoplasm (283). This study provides evidence for an additional role of the multidrug transporter in the development of the fungus. In the fission yeast, *S. pombe*, the MFS drug efflux transporter Caf5 together with a brefeldin A resistance protein-encoding gene, Bfr1 was found to be up-regulated in cells where Int6CT was over expressed (284). Int6CT is a C-terminal fragment of translation initiation factor Int6 that may promote transcriptional activity of Pap1. Pap1 plays a role in oxidative stress resistance and enhancement of HBA2 efflux pump gene expression (285).

In *A. fumigatus* itraconazole resistance is moderated in part by MFS AfuMDR3 (286). Expression of this gene was enhanced following exposure to the azole. As a dermatophyte, *A. fumigatus* infects keratinized cells and keratin-rich tissue. Sulfite efflux is required to provide a reducing environment for keratin breakdown where cystine is converted to cysteine and S-sulphocysteine.
Reduced proteins are more accessible to secreted fungal proteases (287). Sulfite secretion is moderated by a MFS tellurite-resistance/dicarboxylate transporter (TDT) family protein. A study on the transportome of C. albicans has recently been published (288). Of the 95 putative MFS-encoding genes, 22 and 9 representatives represent the DHA1 and DHA2 subfamilies, respectively. Candida strains demonstrate resistance to a variety of antifungal agents. The protein CaMdr1p (BENR) provides resistance to benomyl, fluconazole and methotrexate (289, 290, 291). Analysis of a mutation in MDR1 impairing expression of the gene has demonstrated its importantance in virulence of the fungus (292). Recent studies to elucidate the structural and functional of domains of CaMdr1p utilized both tagging of the protein with green-fluorescent protein (GFP) and alanine-scanning mutagenesis of the transmembrane domain 5 believed to contribute to drug/H⁺ transport (294). These studies demonstrated that this transmembrane domain bearing the conserve motif G(X₆) G(X₃) G(X₃) GP(X₂) G is essential for drug/H⁺ transport. Resistance to fluconazole, cycloheximide, 4-nitroquinolone and phenanthroline has also been shown to be provided by FLU1, TMP1 and TMP2 genes of this family (295, 296). An interesting observation using a variety of clinical isolates of C. albicans suggests over expression of FLU1 did not always correlate with drug resistance (297).

Among the plant pathogens, a novel MFS transporter from Botrytis cinerea, Bcmfs1 has been shown to provide tolerance towards the natural toxins camptothecin and cercosporin as well as fungicides (298). In Mycosphaerella graminicola, the MFS drug efflux transporter, MgMFS1 (DHA14) provides resistance to fungicides and naturally occurring toxins particularly strobilurin and cercosporin (299). Interestingly, in bioassays, virulence of the isolate harboring a disruption in the gene was observed to be similar to the control parent strain. In field studies, expression of the gene was found to be elevated under conditions where a sub-lethal concentration of the fungicide trifloxistrobin was present. (300). These two studies demonstrate that some drug efflux transporters play important roles in survival of the fungus when present outside the host.

**C. Regulation of Efflux Pump Gene Expression**
Recent reviews have discussed regulation of multidrug gene expression in fungi (207, 229). Gulshan and Rowley, 2007 (206) provide an excellent overview of the significant regulatory interactions governing pleitropic drug resistance in S. cerevisiae. Coleman and Mylonakis provide a much-needed overview of drug efflux transporters in plant pathogens and their genomics. Because of its clinical importance and relative ease of genetic manipulation, studies with C. albicans are in abundance. Our understanding of the regulation of ABC transporters in C. albicans has come from studies with clinical isolates showing increased drug resistance and through mutational analyses. The transcriptional regulatory protein Tac1p known to moderate expression of CDR1 and CDR2 genes has been shown to harbor a single point mutation that confers increased drug resistance through these pumps (301). Tac1p has also been shown to play a role in the oxidative stress response and lipid metabolism in part through interaction with its own promoter (302). Transcription factor Ndp80p also regulates Cdr1p (303). A negative regulator Rep1p, first identified in S. cerevisiae has been shown to moderate Mdr1p efflux pump gene expression in C. albicans (262). Over expression of this negative regulator heterologously in S. cerevisiae increased susceptibility to fluconazole. Furthermore, a mutation in the gene encoding the protein in C. albicans enhanced drug resistance. Another regulatory protein Mrr1p (multidrug resistance regulator) has been shown to moderate C. albicans MDR1 gene expression. Clinical isolates of the fungus with increased resistance to fluconazole showed coordinate up-regulation of both the transcription factor and the ABC efflux pump (304).

Analysis of the gene encoding the regulatory protein showed that two point mutations contribute to high-level constitutive expression of the genes those results in increased drug resistance. In C. albicans, MDR1 is under complex regulation that involves the oxidative stress response, drug exposure and multiple transcriptional regulators including Cap1, Mrr1p, Upc2p and Mcm1p (305, 306, 307). Other studies with C. albicans show that uncoupling oxidative phosphorylation in petite mutants of the fungus resulted in reduced sensitivity flucoazole and voriconazole (but no change in the resistance to ketoconazole, itraconazole and amphotericin B) and this phenotype could be attributed to over-expression of MDR1 (292). Recent studies with C. glabrata show that CgCdr1-, CgCdr2- and CgSnq2- encoding genes that moderate azole resistance may not be coordinately
regulated. Gain of function (GOF) mutations in the transcription factor encoding gene CgPDR1 increased azole tolerance *in vitro* through differential expression of CgCdr1-, CgCdr2- and CgSnq2- encoding genes. Furthermore, strains carrying the GOF mutations also showed enhanced virulence in an *in vivo* model when compared to wild type strains (308).

While many studies rely on gene disruptions to assess contributions to a specific phenotype or capability, a recent study with *S. cerevisiae* highlights an important issue related to multiple drug resistance in fungi. Deletion of YOR1 and SNQ2-specific regions resulted in increased efflux in Pdr5p efflux substrates. Additionally, increased transcript production and resistance to Yor1p and Snq1p substrates increased in a PDR5 deletion strain (309).

**D. Identification of Novel and Useful Efflux Pump Inhibitors**

The use of chemicals that can work synergistically with useful antifungal agents can reduce concentrations of the drugs currently used, and possibly reduce the likelihood of exposure-based drug resistance. A 1.8 million member D-octapeptide combinatorial peptide library was recently used to screen for inhibitors of an ABC (Pdr5p) hyperexpressing strain of *S. cerevisiae* that carried deletions in 5 other ABC efflux pumps (310). This study identified a non-competitive inhibitor of ATPase activity and sensitized the strain to fluconazole and increased permeability of the plasma membrane to rhodamine. A naturally occurring compound tetrandrine was shown to increase rhodamine 123 accumulation in *C. albicans* (311). Cerulenin is an inhibitor of fatty acid synthesis and substrate for ABC and MFS efflux transport in *C. albicans*. Structural analogs of the compound were screened for their ability to increase sensitivity to brefeldin A and several were found effective against CaMdr1p-mediated resistance (312). A natural product of turmeric, curcumin, known to block ABC transport activity (ABCB1, ABCC1 and ABCG2) in mammalian cancer cells was shown to effective *in vitro* (313). In these studies, rhodamine 6G (R6G)-efflux was measured in *S. cerevisiae* cells expressing *C. albicans* Cdr1p and Cdr2p proteins. Treatment with curcumin resulted in decreased extracellular R6G in both expressing cell lines, suggesting that the compound impaired efflux pump function. More detailed studies with curcumin also revealed that this compound enhances the effectiveness of certain antifungal products.
(ketoconazole, miconazole and itraconazole) but not others (fluconazole, voriconazole, anisomycin and cyclohexamide). These observations suggest that cucurmin may be a valuable additive when used with conventional antifungal drugs. Curcumin may be also used in more detailed structure/function studies of efflux pumps to identify amino acid residues essential for drug interaction and transport.

E. Biofilms

Microbes are known to form biofilms on surfaces. Biofilms represent a communal aggregate of microorganisms in a matrix with varying amounts of extracellular polysaccharide, protein and nucleic acid. Microbes in biofilms show loss of motility functions, lowered metabolism and enhanced drug and antibiotic resistance (314). The medical importance of biofilms cannot be over emphasized. Biofilms form on in-dwelling medical devices and in wounds. Thus treatment of microbes in biofilms with drugs and antibiotics is a continuous challenge in medicine. A. fumigatus biofilms were studied in vitro and on bronchial epithelial cells for their resistance to a variety of drugs. Azoles were found to be more effective than echinocandins against mature (48 hr) biofilms (315). Overall, decreased anti-fungal drug susceptibility was observed in biofilms than with planktonic cells. Antifungal drug resistance in Candida species has been reviewed recently (316, 317). The role of drug efflux pumps, in biofilms, however, remains somewhat enigmatic (318). Efflux pump mutants (Cdr1p, Cdr2p, Mdr1p) of C. albicans were allowed to form biofilms and were studied with the parental non-mutant at 3 stages (6 hr, 12 hr and 48 hr) for their resistance to fluconazole (319). The results showed that efflux pumps play a role in resistance to this antibiotic in the early but not at the 12 and 48 stage biofilms. In planktonic cells, however, expression of the three pumps was observed at only 12 and 48 hr time points suggesting that in C. albicans, efflux pump gene expression is regulated differentially and in a phase-specific manner. Gene expression of CDR- and MDR-encoding genes was examined in planktonic and biofilm cells of C. albicans and found to be up regulated in biofilms (320). When single and double mutant cells were examined for fluconazole resistance, planktonic cells showed predicted sensitivity but biofilm cells were resistant. These results suggest a multi-factorial based
mechanism of resistance when cells are in a biofilm (320). Research with C. albicans on cell density influence on drug resistance suggests that azole tolerance at high cell densities (found in biofilms) cannot be attributed to drug efflux pumps (321). A strain lacking functional drug efflux pump-encoding genes CDR1, CDR2 and MDR1 were susceptible to azoles at low cell densities but was resistant at high cell densities and when present in a biofilm. Cell wall protein production in C. albicans has been the subject of a recent review and the linkage between cell wall proteins, biofilm formation and drug resistance postulated (322).

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