J. Mol. Microbiol. Biotechnol. (2001) 3(2): 179-184.

Enterococcus faecalis Multi-Drug Resistance Transporters: Application for Antibiotic Discovery

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Abstract

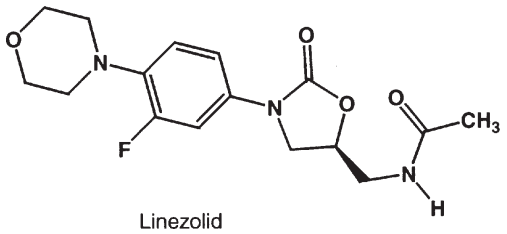
Using bioinformatics approaches, 34 potential multi-drug resistance (MDR) transporter sequences representing 4 different transporter families were identified in the unannotated *Enterococcus faecalis* database (TIGR). A functional genomics campaign generating single-gene insertional disruptions revealed several genes whose absence confers significant hypersensitivities to known antimicrobials. We constructed specific strains, disrupted in a variety of previously unpublished, putative MDR transporter genes, as tools to improve the success of whole-cell antimicrobial screening and discovery. Each of the potential transporters was inactivated at the gene level and then phenotypically characterized, both with single disruption mutants and with 2-gene mutants built upon a Δ *nor*A deleted strain background.

Introduction

Emerging antibiotic resistance, particularly in nosocomial situations, has received wide exposure even in the popular press (Gorman, 1997; Preston, 1999). Awareness has been heightened by the incidence of vancomycin-resistant *Enteroccocus faecalis* (French, 1998) and the recent emergence of a similar resistance in *Staphylococcus aureus* (MMWR, 1997). This observation is particularly significant since Vancomycin was considered to be the last line of defense against Gram positive pathogens. Within the last 6 months, two new Gram positive antibacterials, Synercid® and Linezolid®, have been approved by the FDA. These two agents belong to classes of antibiotics not in prior human therapeutic use and as such could give rise to the hope of a battle won. However, several considerations warn against complacency. Synercid® (quinupristin-dalfopristin) is a combination of two semisynthetic compounds, belonging to the streptogramin class, the first members of which were discovered in 1953 (Charney *et al*., 1953). Virginiamycin, a naturally occurring streptogramin A and B combination, was discovered in 1955 (Somer *et al*., 1957) and has been used as a growth promoter in animal feed until recent years. It has been proposed that this use has selected for virginiamycin-resistant strains of *E. faecium*, which are cross-resistant to Synercid® (Werner *et al*., 1998). Although Synercid® is active against most MLS (macrolide-lincosamide-streptogramin) resistant strains, the existence of a known mechanism directed at this class is cause for concern. Moreover, *E. faecalis* appears to be intrinsically resistant to Synercid® and virginiamycin (Rende-Fournier *et al.,* 1993; Maddock *et al.,* 1999; Lewis and Jones, 2000). In addition, *E. faecalis* resistance and/or superinfection was seen during clinical trials with Synercid® (Blumberg *et al*., 1996; Linden *et al*., 1996).

Linezolid® is the first approved member of a class (oxazolidinones) for which the pharmacophore was reported in 1987 (Slee *et al*., 1987). Despite the fact that this is an exceedingly simple chemical class (Figure 1) and very amenable to modern approaches for combinatorial analog synthesis, it has taken 13 years to bring this drug to market. The rate and extent of the development of resistance to oxazolidinones remains to be seen. However, with few new antibacterials directed towards resistant Gram positive bacteria in the clinic, the identification of new pharmacophores continues to be of urgent importance.

While many mechanisms have been identified as the cause of emergent drug resistance, the expression, or overexpression, of multidrug MDR efflux pumps have been demonstrated to contribute significantly. Antibiotic resistance has been directly attributed to this mechanism in a number of model organisms, as well as important Gram positive pathogens, including *S. aureus* (Yoshida *et al.,* 1990; Hsieh *et al*., 1998), *E. faecalis* (Lynch *et al*., 1997) and *S. pneumoniae* (Gill *et al.,* 1999). In Gram positive pathogens in particular, *pmrA* has been identified as an efflux pump associated with fluoroquinolone resistance *in Streptococcus pneumoniae* (Gill *et al*., 1999) and *mreA* from *Streptococcus agalactiae* has been characterized as a novel macrolide efflux gene (Dib-Hajj *et al*., 1997). These



Linezolid

Figure 1. The chemical structure of Linezolid®

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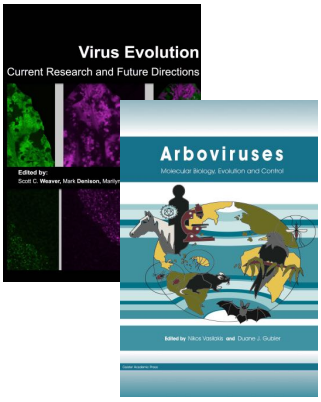

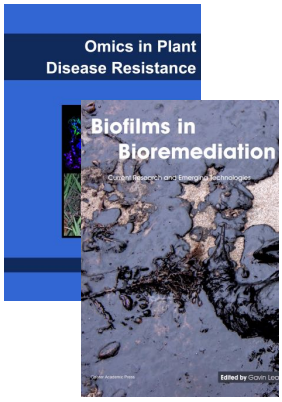

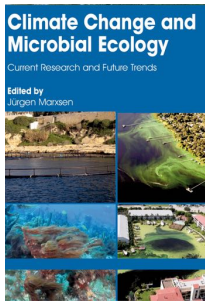

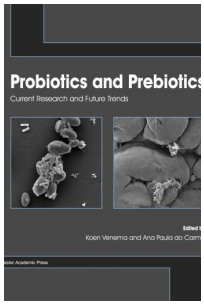

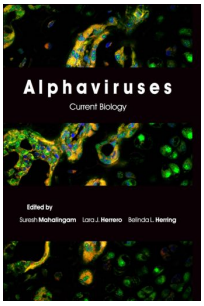

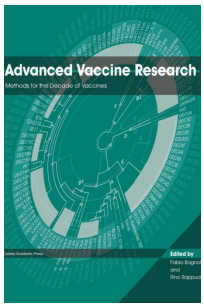

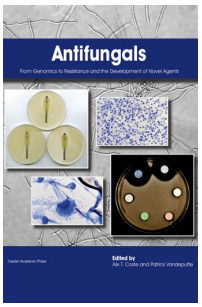

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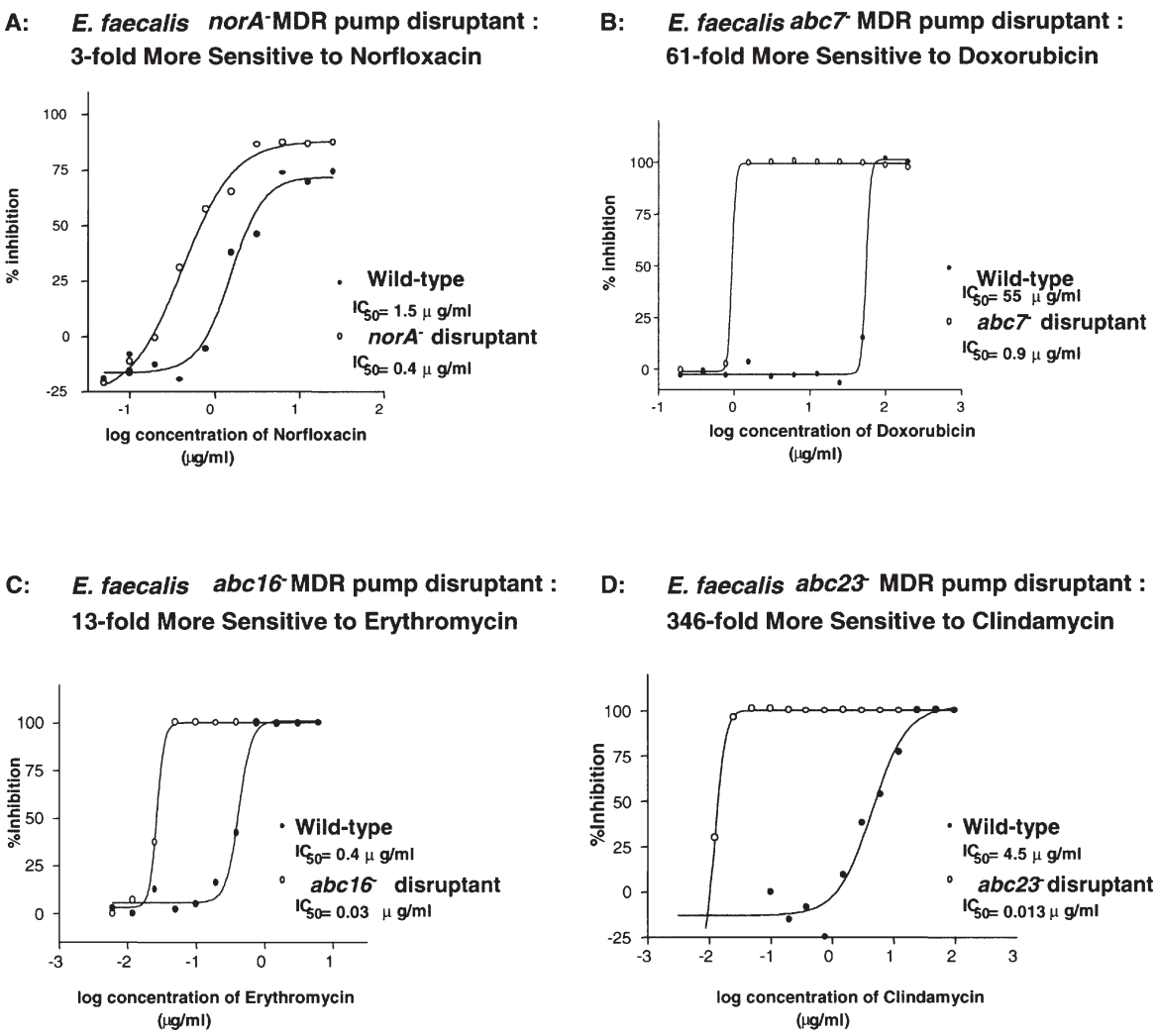

Figure 2. Dose/response curves demonstrate observed hypersensitivities in disruption mutants. Each IC_{50} was calculated using GraphPad Prism software.

export systems can extrude a wide range of structurally dissimilar compounds. (Paulsen *et al.*, 1996; Lewis *et al.*, 1997). Significantly, MDR efflux pumps have been described in phylogenetically diverse organisms including bacteria (Nikaido, 1996), yeast (Kolaczkowski *et al.*, 1998) and mammals (Chen *et al.*, 1986), suggesting their fundamental importance. Many MDR efflux pumps are members of multi-gene families with the best described being the ATP Binding Cassette (ABC) family of membrane proteins, the Multiple Facilitator Superfamily (MFS) of proteins, Multidrug And Toxic compound Extrusion family (MATE), and the Small Multidrug Resistance (SMR) family, (Michaelis and Berkower, 1995; Paulsen *et al.*, 1996, Brown *et al.*, 1999).

A traditional approach to screening for new antimicrobials typically involves the inhibition of growth of a screening strain, preferably of the target pathogen. Such whole-cell antimicrobial assays have the inherent advantage in that advanced knowledge of the mode of action of an antibacterial agent is not required. Hence, such assays are capable of revealing agents with completely novel molecular targets and/or modes of action.

A general problem facing natural product extract-based antimicrobial screening is, that because such extracts are complex mixtures of chemicals, the concentration of any one particular component is undefined, and can be very low. In addition, the effectiveness of screening using whole cells is further diminished by the presence of multiple

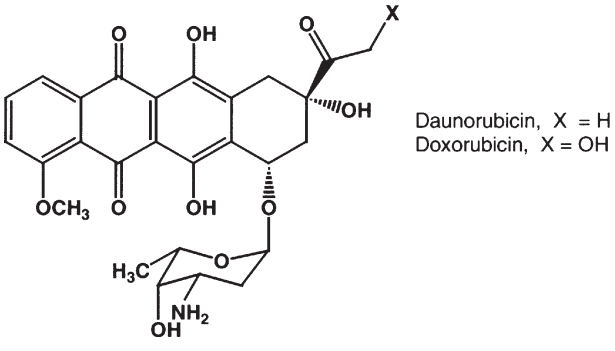

Figure 3. The chemical structure of Daunorubicin and Doxorubicin.

Table 1. *E. faecalis* putative transporter sequences and demonstrated homologies to known multidrug transporters from bacteria, yeasts, and mammals. Shaded boxes represent E value calculations generated by BLAST searching each candidate sequence against GenBank. Unshaded E values represent hypothetical MDR transporters or transporter genes known to efflux metabolites, metals, or other non-antibacterial compounds.

E. faecalis Sequence identifier	E value[a]	*E. faecalis* sequence identifier	E value[a]	*E. faecalis* sequence identifier	E value[a]
ABC1	9.20E-114	ABC9	7.70E-58	MF2	7.80E-62
	4.80E-110	ABC10	2.40E-51		1.80E-46
	2.50E-107	ABC11	5.60E-109		4.60E-43
	3.50E-97		1.30E-97		2.00E-42
	1.80E-90		6.10E-97		1.10E-39
	3.30E-90		2.30E-87		1.40E-34
ABC 2	4.70E-123		1.60E-85		1.40E-32
	5.30E-117	ABC12	3.30E-81		4.60E-30
	6.90E-114		5.90E-81	MF3	3.30E-85
	6.90E-106		4.30E-66		3.80E-27
	9.30E-103	ABC13	6.30E-21		2.60E-22
ABC3	8.40E-106	ABC14	7.20E-92		3.10E-22
	6.30E-57	ABC15	4.60E-27	MF4	2.30E-129
	1.50E-56		1.00E-25		4.10E-95
ABC4,ABC5	1.70E-143	ABC16	3.10E-88		8.80E-49
	2.80E-117		8.20E-31		1.90E-39
	1.80E-115	ABC17	7.10E-62		6.60E-37
	1.40E-104		8.00E-57		4.90E-32
	4.50E-103	ABC18	5.90E-62		4.80E-30
	1.70E-101		2.40E-51	MF5	1.40E-46
	4.90E-96	ABC19	1.00E-46		2.70E-43
	5.20E-73		1.80E-43		7.90E-39
	8.40E-58	ABC20	2.50E-35		5.10E-37
	2.80E-56	ABC21	3.40E-24		3.90E-31
ABC6	6.00E-75	ABC22	5.70E-43		6.20E-15
	8.00E-63		5.00E-40	MF6	1.90E-67
	5.60E-56	ABC23	3.60E-39		4.90E-54
ABC7	5.30E-106	Bmr	7.30E-104		8.30E-29
	6.90E-105	NorA	1.00E-73		1.80E-27
	4.70E-103		1.60E-89		3.60E-26
	6.80E-100		4.60E-92	MF7	4.70E-28
	1.70E-71	MF1	4.00E-59		4.20E-27
	1.40E-57		5.20E-51	Mate 1	1.10E-13
ABC8	4.50E-134		1.40E-48		3.90E-10
	8.20E-115		1.80E-48		9.90E-08
	5.80E-108		9.00E-45		6.00E-07
	1.20E-102		1.40E-39	Smr1	2.60E-24
	4.90E-90		2.20E-34		3.70E-20
	3.10E-71				

[a] The E value relates to the smallest sum probability of the number of hits one can expect to see by chance when searching a database of a certain size. In practice, an E-value threshold of 0.001-0.1 is often considered significant.

multidrug pumps in many bacteria and yeast. In particular, extrusion by MDR pumps of antibiotics present in screening samples at low concentrations significantly decreases the chances of antimicrobial discovery. Thus, there is a strong rationale for increasing the sensitivity of whole-cell screens for new antibiotics. For intracellular target assays, the effective concentration of a test agent is a function of the rates of influx and efflux and the ability of the agent to bind to its target within the cell. Each of these offers opportunity for increasing screen sensitivity. For example, one approach has been to compromise the cell wall and/or cell membrane and hence increase the rate of influx. This is, however, accompanied by an increase in the rate of passive efflux. The prevalence of active efflux via transmembrane pumps provides a more targeted approach to constructing supersensitive whole-cell screening strains. In unpublished work, we have improved the success of whole-cell antifungal screening by using specific strains deficient in a variety of novel MDR-transporter genes. These strains are significantly more sensitive to known antifungal agents and have enabled the discovery of new agents, from both natural product and synthetic chemical libraries, where these agents would not have been discovered using wild type organisms.

Table 2. Table of observed MIC's in insertional knockout strains of *E. faecalis*. The total panel of drugs tested consisted of enoxacin, ofloxacin, norfloxacin, ciprofloxacin, tylosin, erythromycin, spiramycin, clindamycin, lincomycin, virginiamycin, Synercid, chloramphenicol, tetracycline, ampicillin, bacitracin, novobiocin, puromycin, gentamicin, rifamycin, pentamidine, crystal violet, CCCP, acriflavin, daunorubicin, doxorubicin, ethidium bromide, rhodamine 123, and mithramycin.

Gene knockout	Drug[a]	MIC μg/ml		WT/*-gene* ratio
		Wild-type strain	*-gene*	
abc7⁻	Doxorubicin	>100	1.56	64
	Daunorubicin	25	3.12	8
	Ethidium Bromide	12.5	6.25	2
	Ofloxacin	12.5	3.12	4
	Chloramphenicol	6.25	3.12	2
abc11⁻	Pentamidine	250	31.2	16
	Chlorhexidine[b]	12.5	1.56	8
abc16⁻	Erythromycin	0.78	0.1	8
	Azithromycin[b]	1.56	0.1	16
	Clarithromycin[b]	0.39	0.05	8
abc23⁻	Clindamycin	25	0.05	500
	Lincomycin	50	0.2	250
	Virginiamycin M	50	0.78	64
	Synercid	25	0.78	32
Δ*norA*	Ciprofloxacin	1.56	0.78	2
	Norfloxacin	6.25	3.12	2
Bmr⁻	Gentamicin	100	50	2
	Erythromycin	1.56	0.78	2

[a] A total of 28 known, chemically diverse antimicrobial agents were tested. None of the 24 other single gene disrupted strains created consistently showed more than 2 fold increased sensitivity (compared to the parent) towards any of the 28 test substances above.

[b] Compounds indicated were not tested against all of the single-gene disruptants. They were tested only against the specific gene disruption strain that initially was susceptible to a related chemical compound.

Putative MDR Transporter Identification

Active efflux has previously been demonstrated in *E. faecalis* by studying the effect of energy (glucose fed versus glucose starved) on the accumulation of labeled norfloxacin, chloramphenicol, tetracycline and benzyl penicillin by wild-type strains. (Lynch *et al.*, 1997). Individual pumps were not characterized, nor was any genetic analysis part of this work. In our study, we used the amino acid sequence and membrane topology of several known MDR transporters to probe, using a series of BLAST searches for homologs, in the TIGR unannotated *E. faecalis* database (www.TIGR.org) (Altschul *et al.*, 1990; Worely *et al.*,1995, 1998). From this, an MDR pump gene candidate list of 23 ABC superfamily homologs, 9 MF family homologs, 1 MATE homolog, and 1 SMR homolog was generated (Table 1). All candidate sequences were retrieved from the unannotated database and examined for open reading frames. The 23 ABC transporter candidates were also analyzed for predicted transmembrane regions using SOSUI (Hirokawa *et al.*, 1998) through the BCM search launcher (Smith *et al.*, 1996) and predicted ABC family signature sequences and ATP-binding motifs using Motif finder (Hofmann *et al.*, 1999; www.motif.genome.ad.jp). Candidates containing 4-6 predicted transmembrane regions, ABC signature sequences and ATP-binding motifs were placed at a high priority. MF candidates were also examined for predicted transmembrane regions and were excluded if they did not meet the arbitrary criteria of having greater than nine of these regions.

We initiated a comprehensive functional genomics study aimed at generating site-directed insertional mutagenesis to inactivate each individual gene listed in

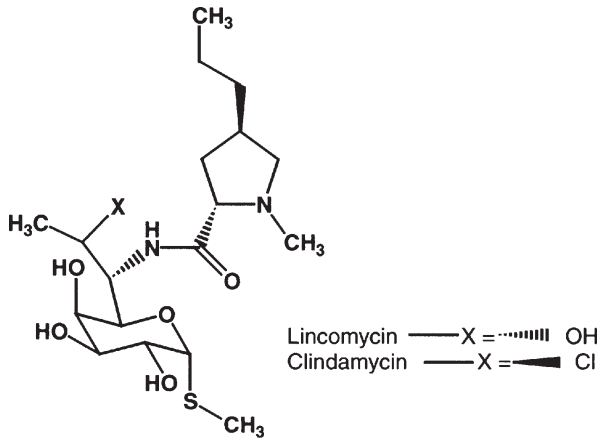

Figure 4. The chemical structures of Lincomycin and Clindamycin.

Table 1. For these studies, a 500-700 base pair internal fragment from the N-terminal end of each target gene was recovered from genomic DNA (*E. faecalis* OG1RF), cloned into vector pTEX4577 and the plasmids were electroporated (Qin *et al*., 1999) into OG1RF and selected for transformants on BHI agar supplemented with kanamycin. Transformants were individually confirmed by PCR to have the targeted insertion of each plasmid. Once confirmed, each single-gene disruption mutant strain was tested against a panel of 28 compounds using a microbroth dilution assay (NCCLS document M7-A5) and its susceptibility compared to that of the wild-type.

The TIGR sequence database was generated from vancomycin-resistant *E. faecalis* strain, V583. Although we amplified candidate sequences *abc9*, *abc12*, *abc20*, and *smr1* from this strain, we were unable to amplify them after several attempts from working strain OG1RF. The apparent absence of these genes from OG1RF may be explained by strain to strain variation.

Novel Transporters and Observed Substrates

The observed phenotypes of these strains functionally implicated several genes as responsible for drug efflux in *E. faecalis*. In particular, the *abc7*$^-$ single-gene disruption mutant has shown a significant hypersensitivity to daunorubicin, doxorubicin, and ofloxacin. Ethidium bromide and chloramphenicol showed a 2-fold increase in susceptibility, which given the inherent variability of an MIC-based assay can only be regarded as significant in that it was a consistent result from several independent measurements. Figure 2B illustrates an approximately 60-fold increase in hypersensitivity for doxorubicin against the *abc7*$^-$ strain compared to the wild-type OG1RF (Table 2). Although conventional wisdom dictates that these MDR pumps are generally promiscuous and accept a wide variety of substrates, these data suggest that this may be a misconception arising from the large number of such pumps present in each organism. For example, the ABC7 transporter exhibits a high degree of specificity within a structural type. The increased sensitivity of strains with disabled ABC7 to the two anthracyclines, daunorubicin and doxorubicin is significantly different (8 and 60-fold, respectively) despite the fact that they differ in structure only by a single hydroxyl group. (Figure 3)

Similarly, the *abc23*$^-$ disruption strain has shown significant hypersensitivity to clindamycin, lincomycin, virginiamycin and Synercid® (Table 2; Figure 2D). Coincidentally, the lincosamides and the streptogramins (virginiamycin and Synercid® exert their antibacterial activity by binding to the bacterial 50S ribosomal sub-unit and inhibiting protein synthesis even though they have unrelated chemical structures (Figures 4 and 5). The two components of the streptogramins are thought to act synergistically by binding to two separate sites of the bacterial 50S ribosomal subunit. Synercid® is a sterile lyophilized formulation of two semi-synthetic pristinamycin derivatives, quinupristin (derived from pristinamycin I) and dalfopristin (derived from pristinamycin IIA) in the ratio of 30:70 (w/w) (http://www.aventispharma-us.com). Lincomycin and clindamycin also inhibit protein synthesis by blocking peptidyl transferase activity of the 50S ribosomal subunit (Gale *et al*., 1972).

Resistance to streptogramins can develop by multiple mechanisms including; 1) modification of the drug target, 2) inactivation of the drug, and 3) impaired permeability from active efflux or production of altered permeases (Quinupristin/Dalfopristin Drug Monograph, 1998). The most commonly observed of these is the modification of

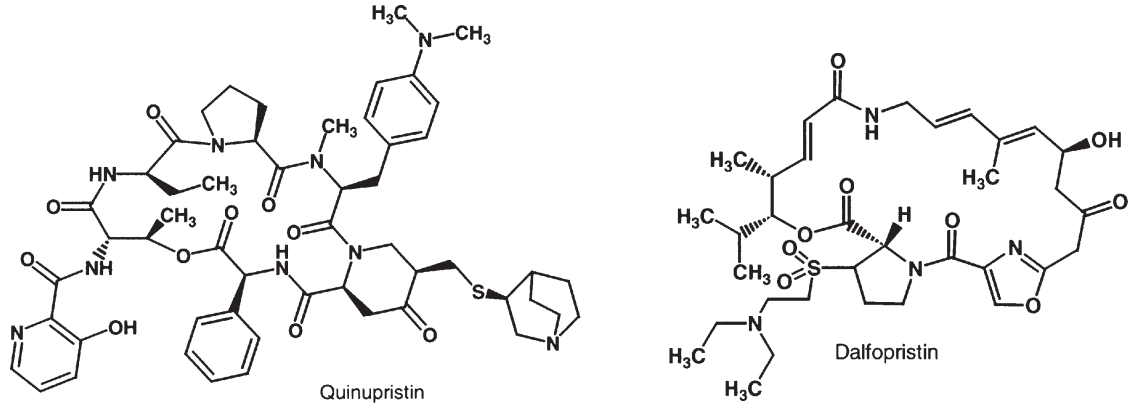

Figure 5. Synercid® consists of two semisynthetic pristinamycin derivatives (quinupristin and dalfopristin) in a 30:70 (w/w) ratio.

the target, which is mediated by the *erm* gene. This gene encodes an RNA methylating enzyme that results in reduced binding of macrolides, lincosamides, and streptogramin B antibiotics (MLS_B) (Quinupristin/ Dalfopristin Drug Monograph, 1998). Group A streptogramin antibiotics such as dalfopristin are not affected by this type of resistance. In *E. faecium*, resistant isolates have been associated with the presence of *vat*(E) *(satG)* or *vat*(D) *(satA)* genes responsible for hydrolysis or acetylation of quinupristin and dalfopristin, and studies suggest that this is not the only mechanism (Soltani, *et al.*, 2000). It is likely that the *abc23* gene identified in *E. faecalis* is responsible for transporter-mediated resistance in this organism and could explain why *E. faecalis,* in general, is intrinsically resistant to streptogramins.

Multiple Transporter Knockouts

S. aureus mediates resistance to norfloxacin by expression of the *norA* gene which codes for an MDR transporter. The OG1RF strain of *E. faecalis* was constructed in which a homologous gene had been removed and examined for its susceptibility to a panel of antimicrobial agents. In *E. faecalis*, the Δ *nor*A strain was only 2 to 3-fold more susceptible to norfloxacin (Figure 2A) and it showed little increased sensitivity to ciprofloxacin. Similarly, in *S. aureus*, a *nor*A$^-$ strain was only 4-fold more sensitive than the wild-type to norfloxacin (Hsieh *et al*., 1998). Nonetheless, we reasoned that NorA may still recognize and transport agents, but if these were substrates for other transporters, the effect of deleting *nor*A on the intracellular concentration could be negligible. If a drug is the substrate for multiple transporters, maximal intracellular concentration can only be achieved by inactivating all pumps recognizing the drug and, *a priori,* one would expect that sequential inactivation of the transporters would be synergistic, with the greatest phenotypic effect observed when the last transporter is removed. Starting with the *nor*A deletion strain, we created a series of 2-gene mutants employing the methodology used above. The sensitivity of these to a panel of antimicrobials exactly matched the susceptibility profile of each of the single gene disruptants described in Table 2. This result suggests that disruption of 2 transporters may be insufficient to produce a significant change in drug sensitivity. Lee and co-workers have studied the effects of differential multiple transporter expression in Gram negatives, (*E. coli and P. aeruginosa*). (Lee *et al.,* 2000). Here the situation is more complex in that there are two types of transporters; single component transporters which extrude substrates from the cytoplasm to the periplasm and multi-component pumps which take substrates all the way from the cytoplasm to outside the cell. They concluded that transporters of different structural types were synergistic in their effect, whereas transporters of the same type are essentially additive and explained this with the two sequential pool model of the Gram negative cell. Under this model one might assume that the situation for multiple gene inactivations in Gram positives would approximate the effects seen there from transporters of the same type. Work to produce strains lacking several transporters and examine the effects of this in *E. faecalis* is ongoing.

Conclusions

A total of 34 potential genes coding for efflux pumps were identified *in Enterococcus faecalis* by mining the TIGR database for motifs from published MDR transporter amino acid sequences and transmembrane domains. For 30 of these, we were able to detect and inactivate by insertional mutagenesis the corresponding sequence in *E. faecalis* OG1RF. The phenotypic susceptibilities of the disruption mutants to a battery of 28 structurally diverse antimicrobial agents were characterized and strains *abc7*$^-$, *abc11*$^-$, *abc16*$^-$, and *abc23*$^-$ were significantly more sensitive to at least one of the agents, demonstrating that these gene products are indeed MDR transporters. Significantly, these four ABC insertional mutants revealed large fold-differences that have not been observed in similar studies with other Gram positive organisms. For example, with *L. lactis* LmrA, only minor phenotypic differences in susceptibility were observed when knockouts were analyzed (Ian Paulsen, personal communication). For the remaining gene insertion mutants, no significant drug susceptibility was detected against the battery of compounds tested. More MDR transporters might be identified if the panel of compounds were expanded. It is interesting to note that our work in *E. faecalis* indicates that the dominant efflux pumps present in this organism appear to be ABC transporters, whereas *ad hoc* studies in other bacteria have mainly implicated proton motive force dependent transporters (i.e. QacA, Bmr, MexAB, and AcrAB) (Paulsen *et al*., 1996).
Transporters were identified that play a major role in determining the susceptibility of the organism to streptogramins and lincosamides. NorA, although present in *E. faecalis*, seems to have only a modest effect, even on the susceptibility to norfloxacin. It is possible that other transporters may also pump norfloxacin and may compensate for the deletion of *norA*. MDR disruption mutants hold considerable promise as supersensitive screening strains for the discovery of novel antibacterial pharmacophores. However the large number of MDR transporters present in *E. faecalis* and our results with 2-gene disruptants suggest that several of these transporters will have to be inactivated to achieve a strain that is hypersensitive to a broad structural variety of antibacterial compounds.

Acknowledgements

This work was supported by SBIR Phase I Grant# 1R43 AI44543-01. We would like to thank Aventis for a sample of Synercid®. Preliminary sequence data was obtained from The Institute for Genomic Research website at http://www.tigr.org. Sequencing of *E. faecalis* was accomplished with support from the National Institute of Allergy and Infectious Diseases. In addition, we wish to thank John Cryan for technical assistance.

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