# Antimicrobial Efflux Pumps and Mycobacterium Tuberculosis Drug Tolerance: Evolutionary Considerations

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**Abstract** The need for lengthy treatment to cure tuberculosis stems from phenotypic drug resistance, also known as drug tolerance, which has been previously attributed to slowed bacterial growth in vivo. We discuss recent findings that challenge this model and instead implicate macrophage-induced mycobacterial efflux pumps in antimicrobial tolerance. Although mycobacterial efflux pumps may have originally served to protect against environmental toxins, in the pathogenic mycobacteria, they appear to have been repurposed for intracellular growth. In this light, we discuss the potential of efflux pump inhibitors such as verapamil to shorten tuberculosis treatment by their dual inhibition of tolerance and growth.

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## 1 Drug Tolerance is an Important Barrier to Shortening TB Treatment

The long duration of treatment required with current anti-tuberculous drugs presents a major challenge in tuberculosis (TB) management. At least 6 months of treatment are required to achieve acceptable cure and relapse rates for smear-positive TB (Connolly et al. 2007; Mitchison and Davies 2012). Although an important breakthrough when first introduced, such "short course" therapy is still too long. Adherence to months of TB therapy is difficult, with default rates of nearly 30 % reported in some series (Castelnuovo 2010). The consequences of poor adherence are serious both for the individual patient and for the community: drug resistance, treatment failure, and further TB transmission. Attempts to shorten treatment to 4 months have been thwarted by unacceptably high relapse rates (Johnson et al. 2009).

Why is lengthy treatment with current medications required to cure TB? The answer may be found in observations from landmark TB studies. For years, it has been recognized that when patients with drug-susceptible TB relapse, the bacilli typically remain genetically drug-susceptible and patients respond to their prior treatment regimens (British Medical Research Council 1972; Wallis et al. 1999). Complementary data from early bactericidal activity studies by Jindani and Mitchison demonstrated that during TB chemotherapy, sputum bacillary counts decrease in a characteristic biphasic manner (Jindani et al. 1980). For example with isoniazid, greater than 99 % of the initial sputum bacillary load is killed during the first 2 days of treatment, after which the rate of killing drops off markedly. The residual bacteria are a *phenotypically* resistant, "drug tolerant" population; TB drug minimum inhibitory concentrations are unchanged. Empirical studies have shown that it takes months of therapy to eradicate these bacteria and produce a stable cure (Mitchison and Davies 2012).

The phenomenon of antimicrobial tolerance was recognized in early experiments studying in vitro killing of streptococci and staphylococci by penicillin (Bigger 1944; Hobby et al. 1942) and was subsequently found to generalize to other bacteria, including *Mycobacterium tuberculosis* (Mtb) (McCune and Tompsett 1956; Wallis et al. 1999). Existing models of antimicrobial tolerance differ in their specifics but all invoke the presence of a metabolically quiescent, non-growing population. Older views focused on deterministic mechanisms such

as hypoxia or nutrient starvation, conditions that are thought to occur in the tuberculous granuloma; more recent models implicate stochastic mechanisms whereby so-called "persister" cells arise independently of the growth environment (Dhar and McKinney 2007; Lewis 2010). Although tolerance models that emphasize a role for slow-growing or nongrowing bacteria are compatible with the observation that antimicrobials kill nongrowing TB poorly (Schaefer 1954), evidence from human treatment studies suggest that the drug-tolerant population may not in fact be quiescent. Serial radiological studies have demonstrated that existing lesions may enlarge and new lesions may develop despite an overall efficacious course of therapy, a phenomenon that may be explained by the presence of an enlarging, drug-tolerant Mtb population (Akira et al. 2000; Bobrowitz 1980).

## 2 Actively Growing Intracellular Mycobacteria Exhibit Multidrug Tolerance Mediated by Macrophage-Induced Bacterial Efflux Pumps

Recent insights from the zebrafish-*M. marinum* (Mm) model of TB offer potential explanations for these puzzling radiographic observations. Similar to the expansion of a subset of tuberculous lesions during human therapy, drug-tolerant Mm continue to expand and disseminate within macrophages during infection of zebrafish (Adams et al. 2011). Further investigation with macrophage-like cell lines revealed that subpopulations of both Mtb and Mm become tolerant to multiple classes of antimicrobials including isoniazid and rifampicin upon intracellular residence. The induction of drug tolerance in bacteria by the host macrophage environment has been previously described for *Legionella pneumophila* (Barker et al. 1995) and may be a more widespread phenomenon. Countering prior models, the mycobacterial work revealed that macrophage-induced tolerance is enriched in actively dividing bacteria (Fig. 1) (Adams et al. 2011). This surprising result was explained by the finding that in Mtb, macrophage-induced tolerance to rifampicin is mediated by a bacterial efflux pump, Rv1258c, that also promotes intracellular bacterial growth in the absence of antimicrobials (Table 1) (Adams et al. 2011). Rv1258c, a secondary transporter belonging to the major facilitator superfamily (MFS) of efflux pumps, is structurally related to MefA, a 12-membrane spanning MFS pump involved in macrolide resistance in *Streptococcus pneumoniae* (Ainsa et al. 1998; De Rossi et al. 2002; Li and Nikaido 2009; Saier et al. 2009). Rv1258c is transcriptionally induced following macrophage residence (Table 1) (Schnappinger et al. 2003) and appears to function as a virulence factor induced in the intracellular environment that pathogenic mycobacteria encounter (Chan et al. 2002; Clay et al. 2007; Dannenberg 1993; Ramakrishnan et al. 2000). Its association with rifampicin tolerance appears to be an epiphenomenon, and the identity of the "natural" substrate(s) of Rv1258c remains unknown. Indeed, despite decades of study there are still only a few clearly identified natural substrates of bacterial efflux pumps, such as spermidine in *B. subtilis*, bile

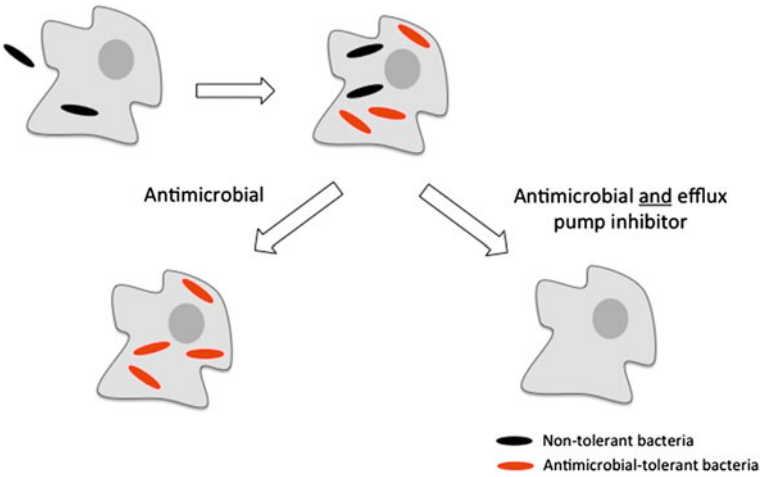


**Fig. 1** *Model for efflux pump inhibitor action in Mycobacterium tuberculosis (Mtb)* Efflux pump expression is induced in Mtb following macrophage residence, perhaps stimulated by macrophage antimicrobial peptides. With antimicrobial treatment alone, nontolerant bacteria are killed, but tolerant bacteria survive and multiply within the macrophage. When antimicrobials are given in conjunction with an efflux pump inhibitor, the tolerant bacteria are killed along with nontolerant bacteria. Note that in this simplified diagram, there is no attempt to differentiate between mycobacterial residence in the cytoplasm versus the phagosome. Modified from (Adams et al. 2011)

salts in *Escherichia coli,* and cyclic-di-AMP in *Listeria monocytogenes* (Thanassi et al. 1997; Woodward et al. 2010; Woolridge et al. 1997).

The findings coupling intracellular bacterial growth and antimicrobial tolerance through induction of bacterial efflux pumps are compatible with the longstanding clinical observation that the duration of curative TB treatment is proportional to the organism burden. Indeed, the highest mycobacterial burden states, smear-positive and cavitary disease, require the longest therapy for a durable cure (Connolly et al. 2007; British Medical Research Council 1989; Zierski et al. 1980). Models of nonreplicating tolerance have attributed this association to high burden disease having increased numbers of nonreplicating as well as replicating bacteria (Connolly et al. 2007). However, the link between mycobacterial burden and treatment duration is equally well explained by the alternative model attributing tolerance to actively growing bacteria. Indeed, this view implicates increased efflux activity as the *driver* of both high mycobacterial burden disease and antimicrobial tolerance; high burden disease states should therefore be enriched in tolerant bacteria.

The role of efflux pumps in promoting drug tolerance opens up a potentially powerful approach for shortening TB treatment. The use of efflux pump inhibitors would target not only bacterial growth, but also drug tolerance. In the laboratory setting, macrophage-induced tolerance is inhibited by verapamil, a calcium channel antagonist in clinical use for years, which has been shown to also inhibit multiple bacterial efflux pumps in vitro (Adams et al. 2011; Marquez 2005; Rodrigues et al. 2011a). Consistent with the observation that macrophage-induced

**Table 1** Macrophage-induced *Mycobacterium tuberculosis* efflux pumps

| Drug efflux pump[a] | Transporter family | Macrophage growth attenuation[b] | Associated drug resistance | Homologs in other mycobacteria | References |
|---|---|---|---|---|---|
| Rv0194 | ABC | Yes | STR | *M. marinum; M. ulcerans* | Braibant et al. (2000), Danilchanka et al. (2008) |
| Rv1218c | ABC | Yes | | *M. smegmatis; M. marinum; M. avium; M. leprae; M. abscessus* | Balganesh et al. (2010, 2012), Braibant et al. (2000) |
| Rv1272c | ABC | Yes | | *M. smegmatis; M.marinum; M. ulcerans, M. avium; M. leprae; M. abscessus* | Braibant et al. (2000) |
| Rv1273c | ABC | Yes | | *M. smegmatis; M.marinum; M. ulcerans, M. avium; M. leprae; M. abscessus* | Braibant et al. (2000) |
| Rv1348 | ABC | ND | | *M. smegmatis; M.marinum; M. ulcerans; M. avium; M. abscessus* | Braibant et al. (2000), Farhana et al. (2008) |
| Rv1349 | ABC | ND | | *M. smegmatis; M.marinum; M. ulcerans; M. avium; M. abscessus* | Braibant et al. (2000), Farhana et al. (2008) |
| Rv1463 | ABC | ND | | *M. smegmatis; M.marinum; M. ulcerans, M. avium; M. leprae; M. abscessus* | Braibant et al. (2000) |
| Rv1687c | ABC | No | | *M. smegmatis; M.marinum; M. ulcerans, M. avium; M. leprae* | Braibant et al. (2000) |
| Rv2686c | ABC | Yes | CIP | *M. smegmatis; M.marinum; M. ulcerans, M. leprae; M. abscessus* | Braibant et al. (2000), Louw et al. (2009), Pasca et al. (2004) |
| Rv2687c | ABC | ND | CIP | *M. smegmatis; M.marinum; M. ulcerans; M. abscessus* | Braibant et al. (2000), Pasca et al. (2004) |
| Rv2688c | ABC | No | CIP | *M. smegmatis; M.marinum; M. ulcerans; M. abscessus* | Braibant et al. (2000), Pasca et al. (2004) |

(continued)

**Table 1** (continued)

| Drug efflux pump[a] | Transporter family | Macrophage growth attenuation[b] | Associated drug resistance | Homologs in other mycobacteria | References |
|---|---|---|---|---|---|
| Rv1258c | MFS | Yes | RIF, OFX, INH | *M. smegmatis; M.marinum; M. ulcerans, M. avium; M. leprae; M. abscessus* | Balganesh et al. (2012), De Rossi et al. (2002), Jiang et al. (2008), Rodrigues et al. (2011b), Siddiqi et al. (2004), Zhang et al. (2005) |
| Rv3239c | MFS | No | | *M.marinum; M. ulcerans* | De Rossi et al. (2002), Louw et al. (2009) |
| Rv3728 | MFS | No | | *M.marinum; M. ulcerans; M. leprae* | De Rossi et al. (2002), Gupta et al. (2010), Louw et al. (2009) |
| Rv1183 (mmpL10) | RND | ND | | *M. smegmatis; M.marinum, M. avium; M. leprae; M. abscessus* | Tekaia et al. (1999) |
| Rv1146 (mmpL13b) | RND | ND | | *M. smegmatis; M.marinum; M. ulcerans, M. avium; M. abscessus* | Tekaia et al. (1999) |
| Rv3065 (mmr) | SMR | ND | ERM | *M.marinum; M. ulcerans, M. avium; M. leprae* | Balganesh et al. (2012), Gupta et al. (2010) |
| Rv0969 (ctpV) | Putative copper exporter | No | | *M. smegmatis, M. marinum, M, ulcerans, M. avium; M. abscessus* | Ward et al. (2010) |
| Rv3578 (arsB2) | Probable arsenic pump | Yes | | *M. smegmatis; M.marinum; M. ulcerans, M. avium; M. leprae* | Ordonez et al. (2005) |

*INH* isoniazid; *RIF* rifampicin; *OFX* ofloxacin; *CIP* ciprofloxacin; *STR* streptomycin; *EMB* ethambutol; *ERM* erythromycin; *ABC* ATP-binding cassette; *MFS* major facilitator superfamily; *RND* resistance-nodulation cell division family; *SMR* small multidrug resistance family; *ND* not determined

[a] Efflux pumps that are significantly induced (1.3–2.6-fold) at 48 h in naive macrophages (Schnappinger et al. 2003). Those highlighted in bold have been tested experimentally for efflux activity, all others are predicted efflux pumps based on homology to efflux pumps in other organisms

[b] Macrophage growth attenuation from resting or IFN$\gamma$-activated macrophages (Rengarajan et al. 2005)

pumps mediate intracellular survival, verapamil also reduces intracellular mycobacterial growth in the absence of antibiotics (Adams et al. 2011; Martins et al. 2008). This chapter will discuss these findings in the context of a current understanding of antimicrobial efflux in mycobacteria and other bacteria, with an emphasis on teleological, functional, and therapeutic considerations.

## 3 Macrophage-Induced Mtb Efflux Pumps are Virulence Determinants

Although information about the natural substrates of efflux pumps is lacking, extensive in vitro studies have shown that efflux pumps in both prokaryotic and eukaryotic organisms can extrude a variety of toxic agents such as antimicrobials and chemotherapeutic agents (Ho and Kim 2005; Li and Nikaido 2009). Much effort has focused on the role of efflux in antimicrobial resistance (Li and Nikaido 2009). For example, the so-called multidrug resistance (MDR) pumps have been implicated in resistance to structurally diverse antimicrobial agents and contribute to the burden of bacterial drug resistance. Efflux-mediated antimicrobial resistance was initially reported in *E. coli* (Ball et al. 1980; McMurry et al. 1980), but has been subsequently recognized in a wide range of organisms, including the often recalcitrant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Coyne et al. 2011; Li et al. 1995). With the identification of the LfrA pump in *M. smegmatis* as a mediator of fluoroquinolone resistance (Liu et al. 1996), there has been a growing interest in the contributions of drug efflux in mycobacteria (da Silva et al. 2011; Louw et al. 2009). Efflux has also been proposed to account for isoniazid-induced tolerance in Mtb and may be mediated by the isoniazid-induced protein IniA (Colangeli et al. 2005; Viveiros et al. 2002).

Our identification of Rv1258c as a mediator of intracellular growth led us to investigate if mycobacterial efflux pumps are widely used for this critical virulence trait. Mining the published literature reveals that 19 of the 55 annotated efflux pumps in the Mtb genome are transcriptionally induced in macrophages (Table 1) (Camus et al. 2002; Cole et al. 1998; Schnappinger et al. 2003). Of the 12 tested by mutational analysis, 7 are required for intracellular growth (Rengarajan et al. 2005). Thus several macrophage-induced efflux pumps serve nonredundant roles in promoting intracellular growth. Moreover, Mtb efflux pumps not found induced in the 48 hour macrophage infection assay have virulence phenotypes, in a 7-day macrophage infection assay and/or in mouse infection models (Bigi et al. 2004; Curry et al. 2005; Rengarajan et al. 2005; Sassetti and Rubin 2003; Schnappinger et al. 2003). These may represent virulence genes that are induced later in the course of macrophage residence or by specific environments in vivo such as the tuberculous granuloma (Chan et al. 2002; Ramakrishnan et al. 2000). Efflux pumps in Gram-negative bacteria have been linked to multiple virulence functions including gut colonization, and adherence and invasion of cultured cells (Table 2) (Piddock 2006b). It is likely that Mtb pumps participate in similar activities. That Mtb

**Table 2** Bacterial efflux pumps associated with virulence

| Pump family | Organism | Virulence phenotype | Proposed mechanism | Mediates antibiotic resistance | References |
|---|---|---|---|---|---|
| **ABC** | | | | | |
| DrrABC | Mtb | In vivo survival | Localization of phthiocerol dimycocerosate in cell wall | Yes | Camacho et al. (2001), Choudhuri et al. (2002), Sassetti and Rubin (2003) |
| MacAB | *Salmonella enterica serovar Typhimurium* | In vivo survival | May detoxify host-derived molecules | Yes | Nishino et al. (2006) |
| Rv1272c | Mtb | In vivo survival | Unknown | ND | Sassetti and Rubin (2003) |
| Rv1747 | Mtb | Intracellular growth; in vivo survival | Substrate for PknF a serine threonine kinase involved in regulating glucose intake | ND | Molle et al. (2004), Sassetti and Rubin (2003), Spivey et al. (2011) |
| Rv3781 | Mtb | In vivo survival | May be involved in arabinogalactan biosynthesis | ND | Dianiskova et al. (2011), Sassetti and Rubin (2003) |
| **MFS** | | | | | |
| MdrM | *Listeria monocytogenes* | In vivo growth | Secretion of c-di-AMP | Yes | Crimmins et al. (2008), Woodward et al. (2010) |
| MdrT | *Listeria monocytogenes* | In vivo growth | Secretion of c-di-AMP<br>Cholic acid transporter | Yes | Crimmins et al. (2008), Quillin et al. (2011), Woodward et al. (2010) |
| NorA | *Staphylococcus aureus* | Host cell invasion | Unknown | Yes | Aeschlimann et al. (1999), DeMarco et al. (2007), Kalia et al. (2012) |
| NorB | *Staphylococcus aureus* | In vivo survival | Unknown | Yes | DeMarco et al. (2007), Ding et al. (2008) |
| P55 (Rv1410c) | Mtb, *Mycobacterium bovis* | Intracellular growth; in vivo survival | Preservation of cell wall | Yes | Bianco et al. (2011), Ramon-Garcia et al. (2009), Rengarajan et al. (2005), Sassetti and Rubin (2003) |

(continued)

**Table 2** (continued)

| Pump family | Organism | Virulence phenotype | Proposed mechanism | Mediates antibiotic resistance | References |
|---|---|---|---|---|---|
| QacA | *Staphylococcus aureus* | In vivo persistence | Increased membrane fluidity | Yes | Bayer et al. (2006), Dhawan et al. (1997), Kupferwasser et al. (1999) |
| Rv0037c | Mtb | Intracellular growth | Unknown | ND | Rengarajan et al. (2005) |
| Rv0849 | Mtb | Intracellular growth | Unknown | ND | Rengarajan et al. (2005) |
| Tap (Rv1258c) | Mtb | Intracellular growth | Unknown | Yes | Adams et al. (2011), Ainsa et al. (1998), Balganesh et al. (2012), Sharma et al. (2010), Siddiqi et al. (2004) |
| **RND** | | | | | |
| AcrAB | *Escherichia coli, Francisella tularensis, Klebsiella pneumoniae, Salmonella enterica serovar Typhimurium, Enterobacter cloacae* | In vivo survival | Efflux of bile acids | Yes | Bina et al. (2008a), Blair and Piddock (2009), Buckley et al. (2006), Helling et al. (2002), Ma et al. (1995), Padilla et al. (2010), Perez et al. (2012), Rosenberg et al. (2003), Thanassi et al. (1997) |
| BesC | *Borrelia burgdorferi* | In vivo survival | Possible component of type I secretion system | Yes | Bunikis et al. (2008) |
| BpeAB-OprB | *Burkholderia pseudomallei* | Host cell invasion | Quorum sensing | Yes | Chan and Chua (2005) |
| CmeABC | *Campylobacter jejuni* | In vivo colonization | Efflux of bile acids | Yes | Lin and Martinez (2006), Lin et al. (2003), Martinez and Lin (2006) |
| MexCD-OprJ | *Pseudomonas aeruginosa* | In vivo survival; hyperexpression compromises expression of type III secretion genes | Secretion of quorum sensing molecules | Yes | Join-Lambert et al. (2001), Linares et al. 2005) |

(continued)

**Table 2** (continued)

| Pump family | Organism | Virulence phenotype | Proposed mechanism | Mediates antibiotic resistance | References |
|---|---|---|---|---|---|
| MexEF-OprN | *Pseudomonas aeruginosa* | In vivo survival; hyperexpression compromises expression of type III secretion genes | Secretion of quorum sensing molecules | Yes | Frisk et al. (2004), Join-Lambert et al. (2001), Kohler et al. (2001), Lamarche and Deziel (2011), Linares et al. (2005) |
| MmpL7 | Mtb | Intracellular growth; in vivo survival | Translocation of phthiocerol dimycocerosate to cell wall | Yes | Camacho et al. (2001), Domenech et al. (2005), Lamichhane et al. (2005), Pasca et al. (2005), Rodrigues et al. (2011b); Sassetti and Rubin (2003) |
| MmpL10 | Mtb | In vivo survival | Unknown | ND | Lamichhane et al. (2005), Sassetti and Rubin (2003) |
| MtrCDE | *Neisseria gonorrhoeae, Neisseria meningitidis* | In vivo survival | Resistance to host antimicrobial defenses | Yes | Hagman et al. (1995), Jerse et al. (2003), Shafer et al. (1995, 1998), Tzeng et al. (2005), Warner et al. (2008) |
| VexH | *Vibrio cholerae* | Intestinal colonization | Export of cholera toxin and toxin coregulated pilus | Yes | Bina et al. (2008b), Taylor et al. (2012) |
| TolC | *Brucella suis, Francisella tularensis, Legionella pneumophila, Salmonella enterica serovar Typhimurium, Salmonella enteritidis* | Intracellular growth; intestinal colonization | Might be involved in efflux of reactive oxygen species | Yes | Buckley et al. (2006), Ferhat et al. (2009), Nishino et al. (2006), Platz et al. (2010), Posadas et al. (2007), Stone and Miller (1995), Wu et al. (2012) |
| **Other** | | | | | |
| ArsB2 | Mtb | Intracellular growth | Probable arsenic pump | ND | Rengarajan et al. (2005) |
| CopA | *Neisseria gonorrhoeae* | Invasion and survival | Export of copper ions | ND | Djoko et al. (2012) |
| CtpV | Mtb | Intracellular growth; in vivo survival | Putative copper exporter | ND | Rengarajan et al. (2005), Ward et al. (2010) |

*Mycobacterium tuberculosis* (*Mtb*)

allocates a great number of efflux pumps to ensure intracellular survival is consistent with it being a central strategy for mycobacterial virulence (Cosma et al. 2003; Shepard 1957).

## 4 Mtb Macrophage-Induced Efflux Pumps: Signals and Substrates

The presence of distinct host-generated defenses within the macrophage may explain the observation that multiple macrophage-induced Mtb pumps are individually essential for intracellular growth. What are the stimuli that induce pump expression and what are the substrates? Do different pumps have shared stimuli but unique substrates? Although these answers are not clear, indirect clues suggest that tolerance-producing mycobacterial efflux pumps may be induced by antimicrobial peptides (AMPs) (Adams et al. 2011). Indeed, macrophage-induced *M. marinum* tolerance is not inhibited by dexamethasone, a glucocorticoid that reduces most macrophage defenses while sparing antimicrobial peptide expression (Adams et al. 2011; Duits et al. 2001; Ehrchen et al. 2007). This model has precedence: the macrophage-derived AMP LL-37 induces transcription of *mefE*, which encodes one component of a *Streptococcus pneumoniae* efflux pump related to Rv1258c (Zahner et al. 2010).

Could AMPs also be substrates of the macrophage-induced efflux pumps? Studies predominantly from Gram-negative bacteria suggest this could be the case (Bengoechea and Skurnik 2000; Brissette and Lukehart 2007; Padilla et al. 2010; Shafer et al. 1998; Tzeng et al. 2005; Warner and Levy 2010). For example, in *Neisseria gonorrhoeae,* mutation of the Mtr efflux pump and treatment with the chemical efflux pump inhibitor carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP) both increase AMP sensitivity as well as intracellular AMP accumulation (Shafer et al. 1998). Similar to the Rv1258c pump, Mtr also confers antibiotic resistance (Hagman et al. 1995). While Rv1258c mediates tolerance to the hydrophobic antibiotic rifampicin but not the hydrophilic isoniazid, Mtr similarly mediates resistance to rifampicin and the hydrophobic erythromycin, but not the hydrophilic antibiotic streptomycin. Though many pumps have been noted to have broad substrate promiscuity (Neyfakh 2002), these examples suggest that hydrophobic compounds may be transported by a more limited subset of pumps.

Of course, other mechanisms likely contribute to AMP resistance aside from AMP efflux (Kraus and Peschel 2006). While a comprehensive screen of *Neisseria meningitidis* mutants with increased susceptibility to an AMP-like cyclic lipopeptide revealed a predominance of mutations in the *mtr* gene, mutations in other genes involved in lipid A and pilin synthesis were also identified (Tzeng et al. 2005). In addition, *S. aureus* strains overexpressing the QacA pump showed evidence of decreased membrane fluidity (Bayer et al. 2006). Thus, it would appear that resistance to AMPs can be mediated directly through efflux pumps as well as

by compensatory mechanisms such as cell surface remodeling. In this context it is interesting that MmpL7, an Mtb efflux pump required for intracellular survival, is thought to exert its virulence effects by transporting phthiocerol dimycocerosates (PDIM) into the bacterial cell wall (Camacho et al. 2001; Cox et al. 1999). Similarly, a role in compensatory cell wall remodeling rather than direct drug transport may explain IniA's contribution to tolerance to isoniazid and ethambutol, drugs that act on the mycobacterial cell wall (Colangeli et al. 2005).

A consideration of the signals and substrates of the macrophage-induced Mtb pumps must account for two observations. First, only a subpopulation of intracellular bacteria exhibits antibiotic tolerance. The most likely explanation for this finding is that there is variation in efflux pump expression, with higher-expressing organisms attaining a drug-tolerant, macrophage growth-adapted phenotype. Why might pump expression vary? Variation could be stochastic or might occur if the pump inducing signal is accessible to only a subset of bacteria, such as the subpopulation of Mm and Mtb that exit the phagosome into the cytosol (Stamm et al. 2003; van der Wel et al. 2007). However, Mm lacking RD1/ESX-1, which is required for cytosolic translocation (Simeone et al. 2012), still become drug-tolerant after macrophage residence (Adams et al. 2011); pump inducing signals must therefore be present in the phagosome. Of note, AMPs are known to access phagosomal bacteria; the macrophage-derived cathelicidin LL-37 has been shown to effectively kill Mtb in cell culture (Liu et al. 2006, 2007).

Second, in advanced human TB, most bacteria reside in the granuloma's necrotic core, known as the caseum (Canetti 1955; Grosset 2003). However, the effects of macrophage-induced tolerance may still be relevant after Mtb has exited macrophages. Down-regulation of efflux pumps may occur relatively slowly and may be balanced by an influx of "freshly tolerant" Mtb brought in by phagocytes that traffic into and lyse within the necrotic caseum (Cosma et al. 2004; Dannenberg 2003). Alternatively, the original stimulus may persist after macrophage lysis; in support of this hypothesis is the finding that macrophage-induced tolerance lasts for at least 5 days in vitro following macrophage lysis (Adams et al. 2011). Finally, additional stimuli in the extracellular environment could also maintain tolerance. Again, AMPs remain viable candidates as they can be produced by a diversity of cell types, including the respiratory epithelium (Parker and Prince 2011).

## 5 Function and Regulation of Macrophage-Induced Efflux: A Teleological Perspective

It is remarkable that the majority of the Mtb macrophage-induced efflux pumps, including those demonstrated to mediate intracellular growth, are widely conserved among mycobacteria with divergent lifestyles, ranging from the environmental *Mycobacterium smegmatis* to the ultimately host-adapted *Mycobacterium leprae* that is incapable of axenic growth (Tables 1 and 3) (Cole et al. 2001;

Tsukamura 1976). Regulation of these pumps may also be conserved, as seen with Rv1258c. In Mtb, its expression is under the transcriptional control of WhiB7, which belongs to an ancient and highly-conserved family of transcriptional regulators found in multiple actinomycetes including the soil-dwelling *Streptomyces*, *Nocardia*, and both environmental and pathogenic mycobacteria (Morris et al. 2005). WhiB7 mediates the characteristic low-level intrinsic resistance of *Streptomyces* and mycobacteria to antimicrobials of multiple classes (Morris et al. 2005). It is induced in response to sub-inhibitory concentrations of antimicrobials and mediates Rv1258c transcription in these settings. Furthermore, WhiB7 is itself induced by macrophage residence (Larsson et al. 2012; Rohde et al. 2012), and would also be predicted to be required for Rv1258c transcriptional induction and bacterial survival in this context.

Despite the varied environments different mycobacterial species face, they may share signals and substrates for efflux pumps. Environmental mycobacteria like *M. smegmatis* may be using pumps to defend against small molecules such as lantibiotics and antibiotics produced by environmental competitors and perhaps enhance growth within free-living amoebae (Asaduzzaman and Sonomoto 2009; Lamrabet et al. 2012). The capacity to extrude AMP-like peptides may have allowed mycobacteria to expand further into intracellular niches, and thereby to a wide range of complex hosts. While the strictly host-adapted bacteria like Mtb and *M. leprae* have not been subjected to environmental antibiotic pressure for millennia, these skills again found use with introduction of antimicrobials into medical practice in the twentieth century.

## 6 Therapeutic Implications for Drug Tolerance

The conservation of these macrophage-induced pumps in a range of pathogenic mycobacteria suggests their inhibition may constitute a therapeutic strategy not only for TB, but for other difficult to treat mycobacterial diseases like leprosy, Buruli ulcer, and pulmonary infections with *M. avium* (Table 3). Indeed, Rv1258c has homologs in these species and rifampicin plays an important part in their treatment (Tables 1 and 3). Multiple drugs—verapamil, reserpine, phenothiazines such as thioridazine, and piperine—have been shown to inhibit bacterial efflux pumps in vitro (Kaatz 2005; Marquez 2005; Rodrigues et al. 2011a; Sharma et al. 2010). In general, the mechanisms by which these agents act are poorly understood. Several models have been proposed, such as: (1) direct binding and inhibition of pump assembly or function; (2) disruption of the transmembrane gradients utilized by secondary transporters; (3) inhibitor binding to the antimicrobial compound; (4) competition for efflux (Marquez 2005; Martins et al. 2008; Pages and Amaral 2009; Piddock 2006a). It is worth noting that some of these efflux pump inhibitors *may also* block macrophage antibiotic efflux, leading to increased intracellular drug levels (Cao et al. 1992), an effect that would potentiate their effect on the bacteria.

**Table 3** Mycobacterial species with homologs of *Mycobacterium tuberculosis* macrophage-induced pumps

| Species (Genome Size)[a] | Environmental Niche | Natural Vertebrate Host | Host Niche | Associated Human Disease(s) | Treatment[b] | References |
|---|---|---|---|---|---|---|
| *M. smegmatis* (7 MB) | Soil | None known | Not applicable | Extremely rare. Case reports primarily of localized disease, e.g. wound infections. | Optimal therapy unknown; resistant to multiple drugs including *rifampicin*. | Long et al. (2012), Pierre-Audigier et al. (1997), Tsukamura (1976), Wallace et al. (1988) |
| *M. marinum* (6.6 MB) | Water Amoebae | Fish and amphibians | Intra and extracellular | "Fish tank granuloma" | Clarithromycin or minocycline or *rifampicin* plus ethambutol | Linell and Norden (1954), Stinear et al. (2008), Yanong et al. (2010) |
| *M. ulcerans* (5.6 MB) | Aquatic insects | None known | Mainly extracellular after brief intracellular phase | Buruli ulcer | Surgery *rifampicin* and streptomycin | Doig et al. (2012), George et al. (1999), Wansbrough-Jones and Phillips (2006) |
| *M. avium* complex (5.5 MB) | Soil and water Amoebae insects, earthworms | Birds, domesticated and nondomesticated mammals | Intracellular | Pulmonary and systemic infections, especially in the immunocompromised. | Pulmonary disease: clarithromycin or azithromycin plus *rifampicin* and ethambutol, with or without an aminoglycoside | Beumer et al. (2010), Biet et al. (2005), Falkinham (2010), Falkinham et al. (2001), Yamazaki et al. (2006) |

(continued)

**Table 3** (continued)

| Species (Genome Size)[a] | Environmental Niche | Natural Vertebrate Host | Host Niche | Associated Human Disease(s) | Treatment[b] | References |
|---|---|---|---|---|---|---|
| *M. abscessus* (5.1 MB) | Water<br>Amoebae | Fish<br>Amphibians | Intra and extracellular | Pneumonia, soft tissue infection, and disseminated infection in the immunocompromised | Multidrug resistant including to rifampicin<br>Pulmonary and disseminated infection unlikely to be cured.<br>Amikacin, imipenem, linezolid, tigecycline retain activity | Medjahed et al. (2010), Nessar et al. (2012), Ripoll et al. (2009) |
| *M. tuberculosis* (4.4 MB) | None known | Humans | Intra and extracellular | Tuberculosis | isoniazid, *rifampicin,* pyrazinamide, and ethambutol | Grosset (2003), Kumar and Rao (2011) |
| *M. leprae* (3.3 MB) | None known | Humans<br>Recently introduced into armadillos | Intracellular | Leprosy | dapsone and *rifampicin*, with clofazimine added for multibacillary disease | Rodrigues and Lockwood (2011), Singh and Cole (2011) |

[a] (Reddy et al. 2009) [b] (Chauty et al. 2007; Mandell et al. 2010)

Verapamil, a calcium channel antagonist long in clinical use, is perhaps the most promising inhibitor for further evaluation as an adjunctive TB agent given its ability to reverse macrophage-induced tolerance to rifampicin (Adams et al. 2011). Other candidates include piperine, a derivative of black pepper that has been proposed to inhibit Mtb efflux pumps including Rv1258c (Sharma et al. 2010; Srinivasan 2007); agents developed to counter Gram-negative efflux pumps such as the Phe-Arg-$\beta$-naphthylamine derivatives (Pages and Amaral 2009); and P-glycoprotein inhibitors originally studied in cancer such as tariquidar (Leitner et al. 2011). The greatest benefit may come from approaches that inhibit multiple pumps, either through broadly-acting inhibitors or a combination of more specific inhibitors.

Could clinically significant resistance to efflux pump inhibitors arise? Certainly these compounds could be vulnerable to many of the same mycobacterial defensive measures used against traditional antimicrobials such as decreased membrane permeability, chemical inactivation of the inhibitor, pump overexpression, pump mutation (Ahmed et al. 1993; Klyachko et al. 1997), or efflux of the inhibitor (Garvey and Piddock 2008). It is possible that the barrier to resistance for efflux pump inhibitors is higher than with traditional antimicrobials. For example, alteration of a binding site on one pump might not be sufficient to confer inhibitor resistance, if the inhibitor can target multiple bacterial pumps involved in tolerance. Moreover, inhibitors that additionally act on macrophage efflux may present a further barrier to evolution of resistance.

## 7 Efflux Pump Inhibition for Drug-Resistant TB

Appreciation for the potential of efflux pump inhibition strategies in drug-tolerant Mtb is recent, but joins a growing interest in developing this strategy for genetically drug-resistant Mtb (Amaral et al. 2010), where current treatment options are limited by even longer duration and increased toxicity (World Health Organization 2011). A substantial proportion of drug-resistant isolates have no identifiable mutations in known resistance-associated genes, and it appears that resistance in some of these isolates may result from increased efflux activity (Louw et al. 2009). In fact, multiple studies have reported increased efflux pump expression in Mtb clinical isolates (Gupta et al. 2006, 2010; Hao et al. 2011; Jiang et al. 2008; Siddiqi et al. 2004). Accordingly, efflux pump inhibitors have been shown to reduce isoniazid, ciprofloxacin, ofloxacin, streptomycin, and linezolid minimum inhibitory concentrations in resistant strains (Escribano et al. 2007; Machado et al. 2012; Richter et al. 2007; Rodrigues et al. 2012; Singh et al. 2011; Spies et al. 2008). Although in vivo data are limited, a promising recent study found that verapamil restored activity of isoniazid, rifampicin, and pyrazinamide against MDR-TB in mice (Louw et al. 2011).

Efflux has been generally associated with low-level intrinsic drug resistance, which may nevertheless exceed clinical breakpoints and can be further amplified by pumps of overlapping substrate specificities (Lee et al. 2000; Piddock 2006b).

Moreover, this resistance may confer a survival advantage during antibiotic treatment that allows other chromosomal mutations to accumulate, further increasing the degree of antimicrobial resistance (Srivastava et al. 2010). Efflux pump activity also appears able to induce cross resistance to structurally and mechanistically diverse compounds: rifampicin treatment of rifampicin-resistant Mtb induced resistance to ofloxacin, which could be reversed with efflux pump inhibitors (Louw et al. 2011). The clinical implications of this phenomenon are quite serious. In areas where access to mycobacterial cultures are limited, the standard TB regiments prescribed to patients with unrecognized drug-resistant TB may not only have minimal efficacy, they may serve to further limit treatment options. Thus, the addition of efflux pump inhibitors to overcome drug-tolerance may have the additional benefit of reducing emergence of genetically drug-resistant Mtb.

## 8 Conclusions

Long recognized as a common mechanism of genetic antimicrobial resistance, efflux pump activity may play a dual role in Mtb, contributing to both virulence and drug tolerance. These pumps may have originally served to defend against environmental toxins that included antibiotics, but came to be utilized by pathogenic mycobacteria for intracellular survival. It is intriguing that their ancient function has come "full circle"—these pumps provide Mtb with a survival advantage in the era of antituberculous chemotherapy. The demonstration of verapamil's activity against macrophage-induced tolerance in the laboratory warrants its assessment in TB patients to determine if it will permit treatment shortening. Further understanding of efflux-mediated drug tolerance may pave the way for new efflux pump inhibitors as well as complementary strategies to kill drug-tolerant mycobacteria.

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