OPINION

Do physiological roles foster persistence of drug/multidrug-efflux transporters? A case study

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Abstract | Drug and multidrug resistance have greatly compromised the compounds that were once the mainstays of antibiotic therapy. This resistance often persists despite reductions in the use of antibiotics, indicating that the proteins encoded by antibiotic-resistance genes have alternative physiological roles that can foster such persistence in the absence of selective pressure by antibiotics. The recent observations that Tet(L), a tetracycline-efflux transporter, and MdfA, a multidrug-efflux transporter, both confer alkali tolerance offer a striking case study in support of this hypothesis.

The introduction of new antibiotics is quickly followed by the appearance of resistance. The problems associated with this resistance are now apparent and have dashed early expectations that the most important battles between pathogenic bacteria and their human hosts are over[1–3].

Antibiotic resistance depends on resistance genes that are either chromosomally encoded or encoded on mobile genetic elements, such as plasmids. These genes can be acquired by horizontal transfer from another strain or after mutational upregulation or modification of existing genes. Antibiotic-resistance proteins confer antibiotic resistance by four main strategies[1,2]: (i) inducing changes in the bacterial surface that reduce the entry of antibiotics; (ii) inactivation of the antibiotic by chemical modification or breakdown; (iii) modification or replacement of the original antibiotic target so that the targeted bacterial function is no longer sensitive to the antibiotic; and (iv) rapid efflux of the antibiotic, preventing an effective level of the compound from reaching its target.

The impressive capacity of bacterial pathogens for self-defence makes it imperative to develop novel antibiotics, a challenge from both scientific and business points of view[1,3,4]. Effective strategies to minimize the burden of antibiotic resistance are also essential. These efforts have focused on reduced and more judicious use of antibiotics to lower the selective pressure for resistance[1,3], with the expectation that this will lower the prevalence of antibiotic-resistant pathogenic strains. However, mixed results have been reported so far[3,5]. This raises the question of whether there are factors other than antibiotic selection itself that promote the persistence of some antibiotic-resistance genes.

A prime candidate for such a factor is a positive physiological impact of the antibiotic-resistance protein that is independent of its role in resistance. Antibiotic-resistance genes could have evolved from existing genes that had other physiological functions in the bacterium, or alternative physiological functions could have been added to an antibiotic-resistance determinant by selection[6–8]. Either way, information about alternative physiological functions could inform strategies for minimizing resistance, but so far there are only a few reported examples of physiological functions for bacterial antibiotic-resistance proteins that are unrelated to antibiotic resistance[9–11]. Therefore, it is striking that two distinct, chromosomally encoded antibiotic-efflux transporters have recently been shown to support pH homeostasis and alkali tolerance[12,13]. One of these transporters is Tet(L), a tetracycline (Tc) efflux protein from the Gram-positive model organism *Bacillus subtilis*[14], and the other is MdfA, a multidrug resistance (MDR) efflux protein from the Gram-negative model organism *Escherichia coli*[15]. The physiological role of these antibiotic-efflux transporters in alkali tolerance presents an interesting case study, with implications for both the emergence and persistence of antibiotic resistance.

Antibiotic-efflux transporters

Bacterial drug and MDR transporters are integral membrane proteins that use cellular energy to actively extrude antibiotics or biocides out of the cell[16]. Most bacteria possess multiple genes encoding such proteins. Some of the transporters encoded are highly drug specific, for example, Tet(L)[17], whereas others, for example, MdfA[15], extrude structurally dissimilar organic compounds and confer resistance to multiple antibiotics. MDR and single-antibiotic-efflux proteins are found among the five main categories of bacterial membrane transporters that have been described on the basis of sequence homology[18,19] (FIG. 1): ATP-binding cassette (ABC) transporters; major facilitator superfamily (MFS) transporters; multidrug and toxic compound exporters (MATE); drug-metabolite transporters (DMTs, which include small MDR (SMR) transporters); and resistance–nodulation–division (RND) transporters.

The ABC pumps are ATPases that use the energy from ATP hydrolysis to pump the drug substrate(s) out of the cell. All the other drug and MDR transporters are

energized by electrochemical gradients of either H^+ (referred to as the proton motive force, PMF) or Na^+ (the sodium motive force, SMF), which are actively maintained across bacterial cell membranes[20] (BOX 1). These transporters couple the free energy that is released from the downhill flux of H^+ or Na^+ (coupling ions) into the cell to the uphill extrusion of the drug(s) against a concentration gradient (FIG. 2). Because the coupling ions and the drug(s) move in opposite directions across the membrane, this type of transport is referred to as an antiport mechanism[21]. Together, the efflux-based resistance mechanisms make a substantial contribution to both the intrinsic and acquired antibiotic resistance of pathogenic bacteria[11,22,23].

Roles of Tet(L) and MdfA

Tet(L) and MdfA use similar antiport mechanisms, although they are distinct in their sequence, topology and substrate specificities (BOX 2). Both of these transporters were first recognized and named for their capacity to actively extrude one or more drugs in exchange for H^+. When encoded in a single copy on the *B. subtilis* chromosome, the contribution of Tet(L) to Tc resistance is demonstrable at low antibiotic concentrations that are probably in the range encountered in natural settings that have not been contaminated by the human introduction of antibiotics[12]. It is probable that MdfA makes a similar contribution to resistance to low concentrations of antibiotic substrates, but a detailed evaluation of the resistance conferred by the single chromosomal copy of *mdfA* has not yet been conducted. It is clear, however, that the resistance conferred by the single chromosomal copies of these genes is far below that observed when *tetL* and *mdfA* are overexpressed on plasmids[12,24,25]. The retention of these genes in the chromosome in strains that are maintained in the absence of antibiotic selective pressure raises the possibility that drug resistance is not their only or main physiological role.

Tet(L) and MdfA in pH homeostasis. A straightforward approach to identify the physiological role of a protein is to change the gene dosage by disrupting the gene or by multicopy expression. For genes encoding antibiotic-efflux proteins, additional physiological roles can then be inferred from the effects of these genetic changes on growth phenotypes other than antibiotic resistance. A random transposition insertion screen for genes encoding Na^+/H^+ antiporters first identified *tetL* as a gene which, when disrupted, leads to alkali- and Na^+-sensitivity[26]. Similarly, a role for *mdfA* in alkali tolerance was found serendipitously during experiments in which its drug-resistance capacities were examined over a range of pH values[27], and MdfA-dependent enhancement of growth at elevated pH was observed in the control assays without drugs. This indication of a role in alkali tolerance is supported by the alkali sensitivity of an *mdfA* deletion strain[13]. Remarkably, *mdfA* expression from a multicopy plasmid enables *E. coli* to grow at pH values as high as pH 10.0 in complex media[13]. This alkaline pH limit is higher than the limit of pH 9.0 for *B. subtilis* expressing Tet(L) from a multicopy plasmid (J. Jin and T.A.K., unpublished data). Both Tet(L)-dependent alkali tolerance in *B. subtilis*[12] and MdfA-dependent growth of *E. coli*[13] at pH ≥9 depend on the presence of Na^+ or K^+ (FIG. 2).

The sensitivity of deletion mutants to alkaline conditions indicates that Tet(L) and MdfA are involved in bacterial pH homeostasis, a property that is essential for all forms of life coping with fluctuating and/or a wide range of external pH values[28–30]. Most bacteria must maintain a cytoplasmic pH close to 7.6 over a range of external pH values (~5.5–9.0) to protect the integrity and activity of cytoplasmic proteins[28,29,31,32]. The capacity for pH homeostasis is best assessed in a pH-shift experiment. Wild-type *B. subtilis* cells can maintain a cytoplasmic pH at or close to 7.5 following a shift of the external pH to 8.5, as long as either Na^+ or K^+ is present. This capacity is lost in a *tetL* deletion strain[12]. In *E. coli*, an *mdfA* mutant shows a considerable decrease in Na^+- and K^+-dependent pH homeostasis[13] above pH 9 when NhaA, the dominant Na^+/H^+ antiporter[33] at pH <9, is not active.

Tet(L) and MdfA as $Na^+(K^+)/H^+$ antiporters. The central role for monovalent cation/H^+ antiporters in pH homeostasis has been shown in several experiments in both prokaryotes and eukaryotes[31–36]. In bacteria, K^+/H^+ and/or Na^+/H^+ antiporters are required for alkali tolerance, as they catalyse the H^+ uptake needed to maintain an optimal cytoplasmic pH[28,29,31,37,38]. As MdfA- and Tet(L)-mediated alkali tolerance requires either Na^+ or K^+, it is plausible that these transporters confer alkali tolerance by $Na^+(K^+)/H^+$ antiport. This idea was tested by assays in membrane vesicles from *E. coli* cells expressing *tetL* or *mdfA* on a multicopy plasmid, and in proteoliposomes containing purified antiporters[13,39,40] (BOX 3). Both Tet(L) and MdfA have Na^+/H^+ antiport and K^+/H^+ antiport activity, as well as the previously shown antibiotic/H^+ antiport activity.

Basics of alkaline pH homeostasis

Bacteria encounter alkaline environments as they pass through the intestinal tracts of human[41] (pH ~8.5) and insect[42] (pH ≥10) hosts. Marine bacteria, which include pathogenic *Vibrio* species, must also tolerate alkaline environments[38]. The external pH in these environments is significantly higher than the optimal cytoplasmic pH of bacteria (pH ~7.6). Therefore, pH homeostasis and the antiporters that support it must

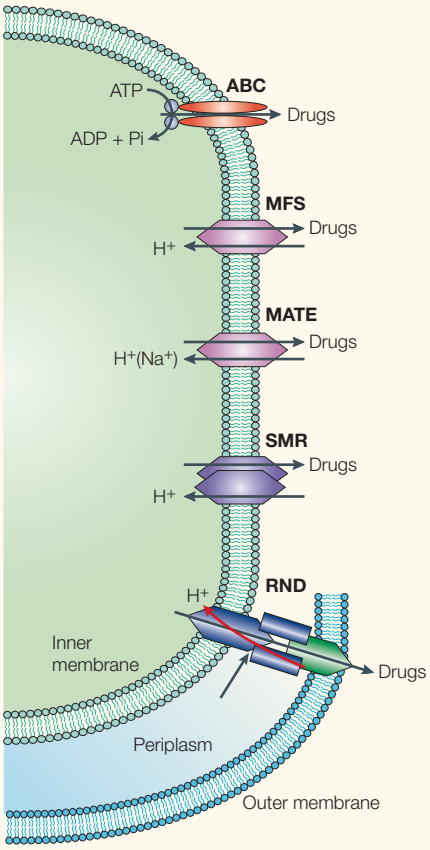

Figure 1 | Bacterial drug- and multidrug-efflux proteins are found in diverse transporter families. ATP-binding cassette (ABC) transporters couple the active extrusion of drugs to the energy of ATP hydrolysis, whereas drug- and multidrug-efflux pumps of the other transporter families (major facilitator superfamily (MFS), multidrug and toxic compound exporters (MATE), drug-metabolite transporters (which include small MDR transporters, or SMRs) and resistance-nodulation-division (RND)) are energized by H^+ or Na^+ electrochemical gradients (the proton motive force or sodium motive force, BOX 1) across the cytoplasmic membrane. The multiple components of RND transporters in Gram-negative bacteria transport substrates out of the cytoplasm across both inner and outer membranes to the medium, and can also extrude substrates outside of the cell directly from the outer leaflet of the inner membrane or from the periplasm[19,79].

Box 1 | **Secondary active transporters**

Electrochemical ion gradients across membranes are usable sources of metabolic energy. By definition, an electrochemical ion gradient consists of two components: an electrical component (Δψ), generated by the flux of a charged species, and a chemical component (ΔpH or ΔNa^+), comprising a chemical concentration gradient of the charged species being pumped in one or other direction across the membrane. Protein complexes in the cytoplasmic membrane of bacterial cells act as primary ion pumps, coupling energy in the form of redox potential, light or chemical bond energy to ion pumping, and this establishes transmembrane electrochemical ion gradients[20].

Most pathogenic bacteria use primary H^+-pumping by respiration or ATPases to extrude H^+, establishing an electrochemical H^+ gradient across the membrane that is termed the proton motive force (PMF) (FIG. 2). The Δψ, or electrical component, of the PMF is inside negative as the positive charge (H^+) is translocated outwards. The ΔpH, or chemical component of the PMF is inside alkaline. The PMF powers ATP synthesis, flagellar rotation, nutrient transport into the cell and toxin transport out of the cell. Many bacteria show comparable generation and use of electrochemical Na^+ gradients (the sodium motive force, SMF).

Membrane-transport proteins facilitate the passage of specific molecules or ions across the otherwise impermeable phospholipid bilayer that surrounds all cells. Genes encoding transport proteins account for 5–10% of bacterial genomes, on average. A large group of these transporters are secondary active transporters, a term chosen because they use the free energy stored in the PMF (functioning secondary to the primary pumps) to drive uphill translocation of substrates (active transport). If the direction of movement of the coupling ion and the substrate are the same during the transport event, the transport is termed 'symport', whereas if the substrate moves in the opposite direction from the coupling ion, the transport is termed 'antiport'. More than 100 families of secondary active transporters have been identified[71].

accomplish net acidification of the cytoplasm, relative to the external milieu. Both *E. coli* and *B. subtilis* have multiple monovalent cation/proton antiporters[33,43,44] (FIG. 2) but they do not contribute equally to pH homeostasis at the alkaline edge of the growth range. This indicates that there are principles that make specific $Na^+(K^+)/H^+$ antiporters especially effective for this function.

One such principle is that to accumulate H^+ in the cytoplasm at alkaline external pH, a Na^+/H^+ antiporter must catalyse an electrogenic translocation reaction across the membrane, not an electroneutral reaction[31–33]. Electroneutral exchange of cytoplasmic Na^+ ions for an equal number of external H^+ ions involves no net flux of charge, therefore, the transmembrane electrical component (Δψ) of the PMF (BOX 1) cannot have a role in catalysing such an exchange. Instead, electroneutral Na^+/H^+ antiport is driven only by the chemical concentration gradient (ΔpH). As the Na^+ cycle in bacteria does not concentrate Na^+ inside cells because Na^+ ions are cytotoxic[45], there is no outwardly directed Na^+ gradient to drive the accumulation of H^+ by electroneutral antiport. Electrogenic exchange involves a stoichiometry of >1 H^+ entering the cell per Na^+ exiting, so that the net positive charge moves inwards during a turnover. Therefore, the Δψ (negative inside relative to the outside) drives H^+ inwards and cytoplasmic acidification can occur. Tet(L) has been shown to catalyse electrogenic Na^+/H^+ as well as K^+/H^+ antiport[39,40,46] (BOX 2).

As bacteria can accumulate K^+ (REF. 47), maintaining a higher cytoplasmic K^+ concentration than is found in the external milieu, the outwardly directed K^+ gradient is an additional option for powering Tet(L)- and MdfA-mediated H^+ accumulation when K^+/H^+ antiport is used. One property of both MdfA and Tet(L) is the high apparent K_m (>10 mM) for their monovalent cation substrates, even though the apparent K_m and K_d for their antibiotic substrates are in the same low-micromolar range found for other drug/H^+ antiporters[13,48,49]. The apparent K_m of MdfA for Na^+ is also at least 50-fold higher than that of two other Na^+/H^+ antiporters from *E. coli*, NhaA and NhaB[44]. The high K_m for the monovalent cation substrates might prevent detrimental loss of cytoplasmic K^+ when it is used as the efflux substrate and the cells possess other Na^+/H^+ antiporters (with a lower apparent K_m for Na^+) that maintain a low cytoplasmic Na^+ concentration[45].

Another property that influences the contribution of a particular antiporter to alkaline pH homeostasis is its overall activity at high pH, a property that varies among different antiporters, even among members of the same antiporter family in different bacterial species[33]. These differences presumably reflect not only electrogenicity but also properties such as pH regulation of antiporter activation, as exemplified by *E. coli* NhaA[50] and *B. subtilis* Tet(L)[39]. Different capacities for H^+ capture at the external surface at high pH can also be important for function at high pH. The special role of Tet(L) and MdfA in the alkali tolerance of *B. subtilis* and *E. coli* respectively, in comparison to the other antiporters (FIG. 2), probably reflects their particular capacity to acidify the cytoplasm at very alkaline pH.

The implications of multiple functions

Emergence and dissemination of antibiotic resistance. The first bacteria to require antibiotic-efflux pumps were the prokaryotic producers of antibiotics. These bacteria make antibiotics to inhibit the growth of other, susceptible bacteria that are competing for nutrients in the same niche. Efflux from the producer organism is necessary for the antibiotic to reach the bacteria that are the intended targets. Antibiotic efflux also protects the producer strain itself from the inhibitory effects of antibiotics that act inside the cell. Genomic evidence further indicates that antibiotic-efflux transporters periodically evolved independently in bacterial targets of the antibiotic, probably from existing host proteins that had other functions[7,51]. Both *in vivo* and *in vitro* evolution experiments provide useful ideas about the paths such evolution can take[6,52]. The proteins that evolved to multifunctionality are hypothesized to already have had modest 'promiscuous activities', that is, extra catalytic capacities that could be mutated to become fully fledged antibiotic-resistance determinants without significant loss of the original function[6]. Antibiotic efflux by both Tet(L) and MdfA is competitively inhibited by the monovalent cation substrates[13,48] (BOX 2), indicating that the antibiotic-efflux capacity could indeed have built on the scaffold of functional antiporters with housekeeping roles in Na^+- and/or alkali-resistance. Extrapolating from the structural characteristics of soluble multidrug-binding proteins, it has been suggested that binding and transport of chemically dissimilar substrates by multidrug transporters involves electrostatic interactions between the substrates and a large hydrophobic pocket of the transporters that has embedded negatively charged residues and conformational flexibility[53,54].

An experiment with Tet(L) supports the idea that drug/H^+ antiport was acquired by an already functional $Na^+(K^+)/H^+$ 12-transmembrane segment (TMS) antiporter, to produce the current 14-TMS version of Tet(L). The two central TMSs (TMS VII and VIII) of Tet(L) are hypothesized to be a late addition to the more common 12-TMS transporter frame[55]. An engineered 'Tet(L)-12' form has been generated by removing those two central TMSs. Tet(L)-12 assembles in the membrane

and catalyses $Na^+(K^+)/H^+$ antiport activities normally, but is devoid of $Tc–Me^{2+}/H^+$ antiport activity[56]. Perhaps the later introduction of the two central TMSs added one or more crucial features (one of which is a particular aspartate residue[56]) that facilitated evolution of the current multifunctional antiporter. The *tetL* gene is only found in some strains of *B. subtilis*, perhaps emerging only in strains that were under antibiotic selective pressure in their environment. The *tetL* gene has probably been part of the *B. subtilis* genome for a long time, given the extent of its integration into the regulatory and physiological networks of this soil bacterium[57]. For example, regulation of *tetL* has a Na^+- and pH-dependent transcriptional component in addition to post-transcriptional regulation[12,58]. MdfA is less integrated into the physiology of *E. coli* than the *tetL* gene is in *B. subtilis*. There is no apparent Na^+- or pH-mediated regulation of *mdfA* and the sequence context surrounding the *E. coli mdfA* gene is most consistent with it having been introduced from an external source. MdfA is also found in pathogenic enteric bacteria of three other genera, *Shigella flexneri*, *Salmonella enterica* serovar Typhimurium and *Yersinia* species, including *Yersinia pestis*[59]. Assuming that the environment of *Y. pestis* in the gut of its insect vector is as alkaline as in many insects[42], it is tempting to hypothesize that *mdfA* has resided in *Y. pestis* longer and might be more integrated into the genomic and physiological contexts of this pathogen than in *E. coli*.

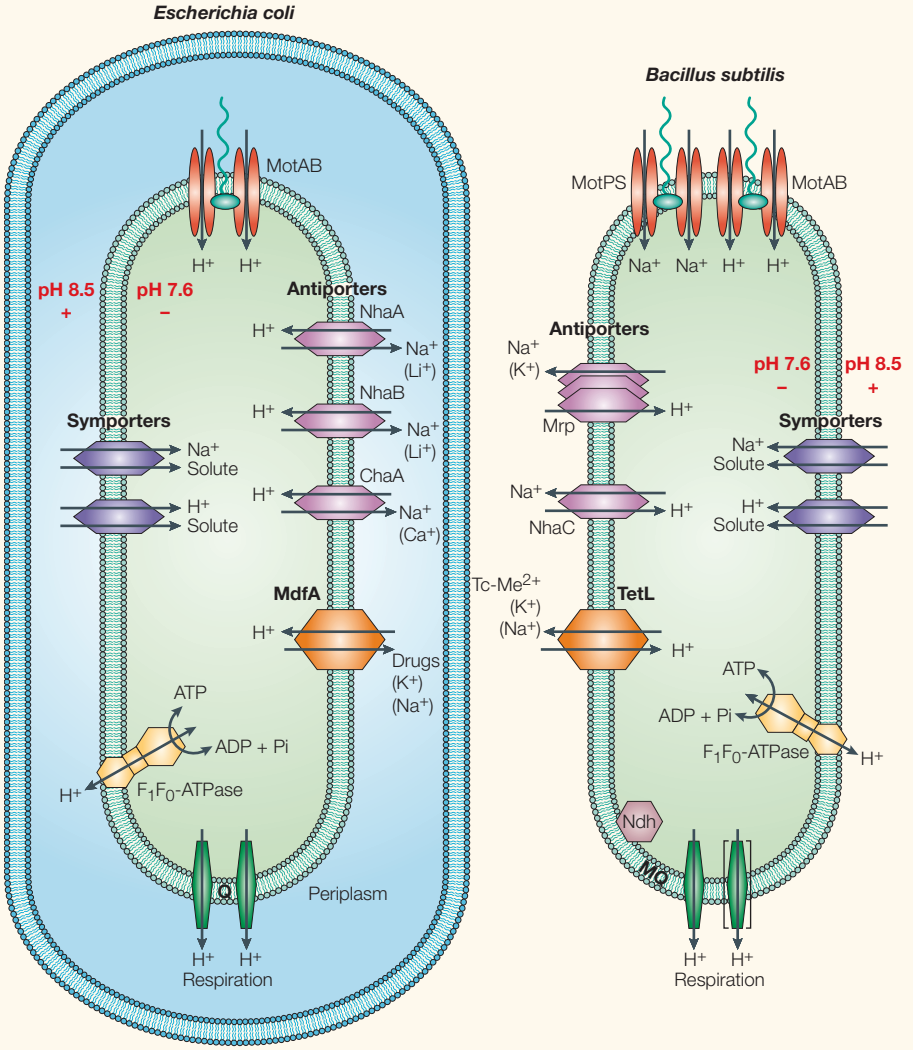

Figure 2 | **Bacterial antiporters.** Many different antiporters participate in the cycles of Na^+ and H^+ that support bacterial pH homeostasis, nutrient uptake, motility and ATP synthesis. Both *Escherichia coli* and *Bacillus subtilis* establish a proton motive force, PMF (BOX 1) through respiration or the H^+-translocating F_1F_0-ATPase. The Na^+/H^+ antiporters establish a sodium-motive force (SMF), using part of the energy of the PMF. Each bacterium has multiple antiporters[33,43,44]. The multifunctional Tet(L) and MdfA transporters are especially important for pH homeostasis at alkaline pH, at which they significantly acidify the cytoplasm relative to the medium. The PMF is also used to energize solute transport, motility and ATP synthesis. Some solute transporters and one of the flagellar motors of *B. subtilis* are coupled to the influx of sodium ions, energized by the SMF[80,81].

Persistence of antibiotic resistance. In the absence of antibiotics, antibiotic-resistance determinants and the plasmids from which they are often expressed generally have negative effects on bacterial fitness because of the metabolic cost of their persistence[60–62]. This is the basis for expecting that a reduction of antibiotic overuse and the consequent selective pressure will promote loss of the resistance determinants. However, this strategy has failed in many instances, raising two main explanations. One explanation is that compensatory mutations arise and eliminate the negative effects of antibiotic-resistance plasmids, as has been documented[60–63]. The second possibility is that physiological functions unrelated to antibiotic resistance can actually foster persistence of resistant determinants in bacteria[8,12]. A beneficial effect of a plasmid-borne resistance determinant has been shown in an experiment that assays the fitness effects of a plasmid carrying both a chloramphenicol-resistance gene (*cat*, encoding a cytoplasmic enzyme that inactivates the drug by modification) and a Tc-resistance gene (*tetC*, encoding a Tc-efflux transporter found mostly in Gram-negative bacteria) on *E. coli*. The plasmid is introduced into a naive, plasmid-free *E. coli* host and into an *E. coli* host that has borne the same plasmid for 500 generations under antibiotic selective pressure before being cured of plasmid. Upon introduction of the plasmid in the absence of the antibiotics, the naive host shows the anticipated reduced fitness, whereas the previously adapted host shows a considerable benefit that relates expressly to the Tc-efflux determinant[61]. It is not yet clear what the specific benefits of the *tetC* gene are in this experiment, but *mdfA* and *tetL* would both be expected to confer benefits upon adapted hosts at elevated pH. For example, MdfA might support the ability of *Y. pestis* to tolerate alkalinity in its insect vector and underpin the alkaline tolerance of *Yersinia enterocolitica* in the intestine and in alkaline foods that it contaminates[64].

The chromosomal antibiotic-resistance genes *tetL* and *mdfA* do not support clinically relevant levels of antibiotic resistance, but their maintenance in the chromosome, because of the physiological fitness benefits, creates a reservoir of antibiotic-efflux genes that enhances persistence of these genes.

Box 2 | **The antiporters Tet(L) and MdfA**

Tet(L) (and the closely related Tet(K) encoded by *Staphylococcus aureus* plasmids[17]) and MdfA belong to the major facilitator superfamily, the largest family of secondary membrane transporters[71,72]. These transporters consist of a single polypeptide chain that crosses the membrane in a 'zigzag' fashion. Tet(L) and Tet(K) (the sequences of which have 55% identity and 74% similarity) have 14 putative transmembrane segments (TMSs)[73]. Tet(L) and Tet(K) only support resistance to tetracycline (Tc) and some of its derivatives. These substrates are transported outwards in complex with a divalent metal ion that has an overall charge[74] of +1 (FIG. 2). Although the precise stoichiometry is unknown, the antiport is electrogenic, indicating that the ratio of inwardly translocated H^+ to outwardly translocated $Tc^{(-1)}$–Me^{2+} is greater than 1 during each turnover. MdfA has 12 putative TMSs and supports resistance to a structurally diverse group of cationic, zwitterionic and neutral drugs, including ethidium bromide, Tc, tetraphenylphosphonium, daunomycin, chloramphenicol and erythromycin. These substrates are extruded without complexing with a metal ion and bear different charges. Interestingly, some of the drug substrates of MdfA are extruded by electroneutral exchange, whereas others are extruded by electrogenic exchange[27].

The monovalent cation/proton antiport activities of both Tet(L) and MdfA probably use the same binding sites and translocation pathway with drug/proton antiport functions. This is supported by the observed competition between the monovalent cation substrates and the drug substrates[13,48], and mutagenesis studies[13,48,75].

In response to the onset of antibiotic pressure, these genes can amplify, as shown for *tetL*[65,66], can mutate their promoter region, as shown for *mdfA*[59], and can also jump into plasmids from which higher levels of antibiotic resistance can be expressed. The *tetL* gene is widely found on mobile elements in Gram-positive pathogens[67,68]. The traffic of antibiotic-efflux genes between the mobile elements and the chromosome is two-way[3].

In principle, information about the physiological roles of antibiotic-efflux transporters could also provide a basis for developing novel inhibitors of related antibiotic-resistance proteins that have clinical relevance, for example, Tet(L) expressed from mobile elements in Gram-positive pathogens[17,67]. The search for novel antibiotics that are not extruded by known efflux transporters (for example, the new generation of glycylcycline tetracyclines) is proceeding side by side with the search for transporter inhibitors that could be used together with antibiotics that would otherwise be extruded[11,69,70]. There is potential for design of Tet(L)- and MdfA-inhibitors based on chemical compounds that inhibit $Na^+(K^+)/H^+$ antiporters. However, although such compounds will surely be of interest for structure–function studies, their utility in a clinical setting will depend on their specificity for prokaryotic antiporters.

Box 3 | **Antiporter assays**

Assays in membrane vesicles are usually conducted in *Escherichia coli* strains that express the test antiporter and have no or low levels of the native antiport reaction[33]. For this assay, cytoplasm-free inside-out (with respect to the cell orientation) membrane vesicles are prepared[76]. They are energized by an electron donor to the respiratory chain, such as NADH or D-lactate, or by ATP for the H^+-pumping F_1F_0-ATPase. Both reactions establish an electrochemical proton gradient (acid and/or positive inside). Radiolabelled antibiotic or cation substrates are then added and, at intervals, samples of vesicles are rapidly separated from the reaction liquid (by filtration or centrifugation methods) and the accumulated substrate is measured. For assays of Na^+/H^+ and K^+/H^+ antiport, a fluorescence assay is widely used. This approach uses a fluorescent ΔpH probe such as acridine orange, the fluorescence of which diminishes (self-quenching) when the dye is concentrated inside the vesicles. The magnitude of the fluorescence quenching reflects the magnitude of the ΔpH formed by respiration. When Na^+ is added, reversal of the fluorescence quenching (dequenching) indicates that protons are exiting the vesicles by a Na^+-dependent mechanism. The apparent K_m of the antiport activity can be estimated from the percentage of dequenching. The limitation of these assays is that their use in cytoplasmic-membrane-vesicles assays falls short of rigorously showing that the observed antiport is attributable only to the test antiporter.

For assays of purified antiporter reconstituted in proteoliposomes, antiporters are extracted with detergent and purified using affinity tags[77]. The purified antiporter is reconstituted into proteoliposomes, sealed phospholipid vesicles in which the antiporter is embedded after the detergent is removed[77,78]. For assays, a ΔpH or a Δψ is artificially imposed, radiolabelled substrate is added and the accumulated substrate is determined at intervals after rapid separation of the proteoliposomes from the reaction mixture. Assays of purified reconstituted proteins show that the purified antiporter protein is solely responsible for the activity.

Future directions

Do the multifunctional properties of Tet(L) and MdfA have positive impacts on fitness in settings that are part of the life cycles of important pathogens? These settings should include alkaline niches in the human and vector hosts as well as niches outside the animal hosts, in ecosystems in which pathogens must survive or grow. The hypothesis that the multifunctional properties of Tet(L) and MdfA have a positive impact on fitness should be tested directly (for example, in competition experiments under conditions of elevated pH and Na^+ that are encountered during the life cycle) and ultimately extended to physiological roles found for other antibiotic-efflux transporters. Fitness effects should be compared in bacteria that are well-adapted to plasmid-borne genes and those that have single gene copies on the chromosome. The detailed delineation of the inter-relationships of *tetL* with the signalling, regulatory and stress-response networks in *B. subtilis* will provide the paradigm of a well-integrated antibiotic-resistance gene, whereas *mdfA* will provide the paradigm of a less physiologically integrated chromosomal drug-efflux gene.

Are functions similar or analogous to those of Tet(L) and MdfA found in other antibiotic-resistance proteins? An established case in point is the clinically important *tetK* gene, which is closely related to *tetL* and can confer similar $Na^+(K^+)/H^+$ antiporter activities when expressed in *Staphylococcus aureus* and other important Gram-positive pathogens[46,48]. When more physiological functions are identified for antibiotic-efflux transporters and their physiological impact is clarified, a rational basis for specific combinations of activities can be developed. Directed evolution approaches will then be suitable for testing the hypothesis *in vitro* from an evolutionary point of view. The insights gained from such studies will hopefully provide a deeper understanding of both the emergence and persistence of antibiotic-efflux transporters.

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Competing interests statement

The authors declare no competing financial interests.

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OPINION

Whither or wither geomicrobiology in the era of 'community metagenomics'

Ronald S. Oremland, Douglas G. Capone, John F. Stolz and Jed Fuhrman

Abstract | Molecular techniques are valuable tools that can improve our understanding of the structure of microbial communities. They provide the ability to probe for life in all niches of the biosphere, perhaps even supplanting the need to cultivate microorganisms or to conduct ecophysiological investigations. However, an overemphasis and strict dependence on such large information-driven endeavours as environmental metagenomics could overwhelm the field, to the detriment of microbial ecology. We now call for more balanced, hypothesis-driven research efforts that couple metagenomics with classic approaches.

Use of modern molecular techniques as applied to investigations in microbial ecology has gained popularity over the last decade. Here, we define molecular techniques as the use of nucleic-acid base sequencing technology to identify the phylogenetic groups present within mixed microbial populations, their community genotypic potentials and the analytical keys for detecting expression of their diverse functional genes.

Molecular approaches can now be considered an essential component of the complete research microbiologist's toolbox, comparable to something as basic as a microscope. A testament to their increased use can be seen by perusing the abstracts from the Tenth International Society for Microbial Ecology meeting, held in Cancun, Mexico (Aug 2004). Of the 1,223 contributed posters, 60% (734) mentioned usage of one or more molecular techniques.

The widespread use of molecular approaches is here to stay, and these technologies are at the cutting edge of science. At least three recent perspectives[1–3] and a scholarly review[4] recount and predict some of the future directions of the rapidly emerging field of environmental genomics as applied to microbial ecology and its subdiscipline of geomicrobiology.

Cultivation of the uncultured

Although estimates of microbial-species diversity vary substantially even in a single sample[5–7], it is generally agreed that only a fraction of the microorganisms that are present have been cultivated[8]. Whereas genomic and 16S rRNA analyses can help to indicate the existence of certain metabolic functions[9,10], a comprehensive understanding of microbial physiologies will undoubtedly require their cultivation. The challenge that environmental microbiologists face today is that conventional enrichment and cultivation techniques cannot be used to cultivate most environmental bacteria. It could be argued that these traditional strategies use conditions that are different from the habitat of many microorganisms and are an important contributing factor to the failure to cultivate most microorganisms in pure culture[11,12]. Therefore, several novel strategies have been developed.

Janssen and co-workers[13] addressed the issue of high concentration of nutrients in media by using a diluted nutrient broth. This approach, together with long incubation times, enabled cultivation of isolates from bacterial groups that are poorly represented in culture collections[14]. A dilution cultivation method for growing bacteria from oligotrophic environments was developed by Button *et al.*[15] Marine microorganisms were diluted to extinction (1 cell per cultivation container) and inoculated into sterilized seawater. After incubation for typically one week, growth of $>10^4$ cells per ml was examined by flow cytometry. This method has been refined[16] with a high-throughput method of Button's protocol now available. Increased sensitivity for the detection of cells based on the use of microtitre dishes and an automated imaging process enabled the isolation of many new planktonic isolates[17,18]. Another approach is based on encapsulation of single cells into microcapsules for parallel cultivation under low organic nutrient flux, followed by flow cytometry to detect and isolate capsules containing microcolonies[19].