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Antibiotic export: transporters involved in the final step of natural product production

Emmanuele Severi* and Gavin H. Thomas

Abstract

In the fight against antimicrobial resistance (AMR), antibiotic biosynthetic gene clusters are constantly being discovered. These clusters often include genes for membrane transporters that are involved in the export of the produced natural product during biosynthesis and/or subsequent resistance through active efflux. Despite transporter genes being integral parts of these clusters, study of the function of antibiotic export in natural producers such as *Streptomyces* spp. remains underexplored, in many cases lagging far behind our understanding of the biosynthetic enzymes. More efficient release of antibiotics by producing cells has potential benefits to industrial biotechnology and understanding the relationships between exporters in natural producers and resistance-associated efflux pumps in pathogens can inform our efforts to understand how AMR spreads. Herein we compile and critically assess the literature on the identification and characterization of antibiotic exporters and their contribution to production in natural antibiotic producers. We evaluate examples of how this knowledge could be used in biotechnology to increase yields of the final product or modulate its chemical nature. Finally, we consider the evidence that natural exporters form a reservoir of protein functions that could be hijacked by pathogens as efflux pumps and emphasize the need for much greater understanding of these exporters to fully exploit their potential for applications around human health.

INTRODUCTION

Antimicrobial resistance (AMR) is one of the most urgent global challenges to human health [1, 2]. The scarcity of effective antimicrobials against multidrug-resistant (MDR) pathogens has led the mainstream media to warn of an 'antibiotic apocalypse' [3], and has compelled the scientific community to direct considerable effort at the discovery of novel molecules able to combat such organisms [4–7].

As new antimicrobials are identified, much academic research strives to detail the biosynthetic steps generating these novel chemistries, in the hope that such understanding can be harnessed for new generations of effective and commercially viable therapeutics [8]. As a result, novel biosynthetic gene clusters (BGCs) for antibiotics are being reported in ever-growing numbers from genome and environmental DNA sequencing projects. The biochemical characterization of these BGCs is often accompanied by efforts to optimize the performance of the BGCs in native or heterologous hosts to increase yields, with studies that unveil the regulation of these BGCs and/or use innovative synthetic biology approaches in order to unlock the full potential of each pathway [9–14].

BGCs often contain genes for exporters [15, 16], integral membrane proteins responsible for the secretion of an enormous variety of molecules including antibiotics (Fig. 1). The vast majority of BGC-linked antibiotic exporters belongs to various subgroups of the ATP-binding cassette (ABC) superfamily and major facilitator superfamily (MFS) of transporters (Fig. 1, Table 1), which while differing by structure, transport mechanism, and mode of energization (Fig. 1) [17, 18], collectively also account for much of efflux-related MDR in nature [19]. While it is commonly assumed that BGC-linked exporters function in the secretion of the antibiotic made by the BGC, the exact nature and importance of their physiological role have been relatively poorly studied and very rarely exploited in biotechnological approaches to improve antibiotic production. Most studies that discover and manipulate BGCs involved strategies that only focus on the biosynthetic genes [20] and do not consider manipulating export as an option.

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Author affiliation: Department of Biology, University of York, Wentworth Way, York, UK.

***Correspondence:** Emmanuele Severi, emmanuele.severi@york.ac.uk

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Abbreviations: ABC, ATP-binding cassette; AMR, antimicrobial resistance; AMS, ABC transporter formaturation and secretion; BGC, biosynthetic gene cluster; MDR, multidrug resistance; MFS, major facilitator superfamily; NBD, nucleotide-binding domain; PCAT, peptidase-containing ABC transporter; RiPP, ribosomally synthesised and post-translationally modified peptides; RND, root-nodulation-division; TMD, transmembrane domain.

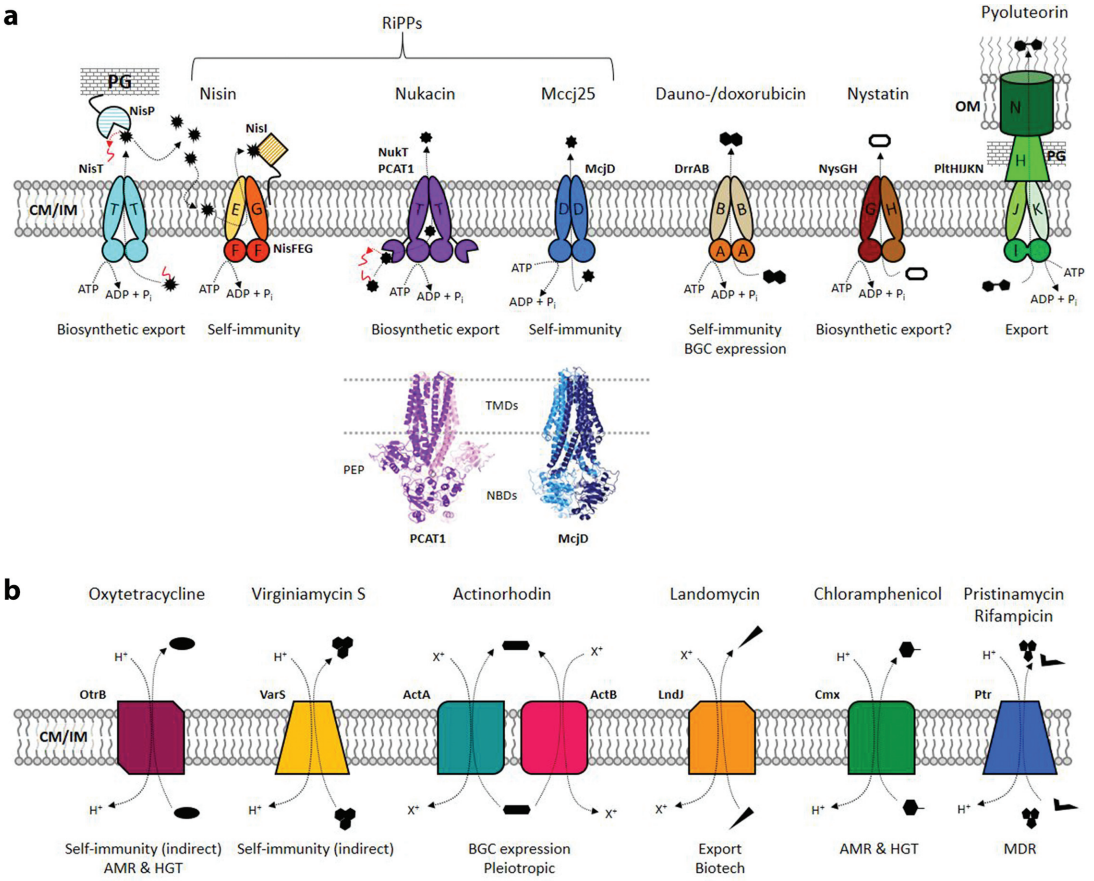

Fig. 1. Diversity of BGC-linked exporters from bacterial antibiotic producers. (a) Examples of antibiotic exporters of the ABC superfamily. Different colours identify distinct transporters and, in order to convey the existing genetic diversity, they also differentiate subunits encoded by separate genes within individual transporters. All these transporters are discussed in the text except for the pyoluteorin exporter PltHIJKN (Table 1), which is here included as an example of envelope-spanning RND exporters from Gram-negative producers. Please note the distinct roles of NisT and NisFEG from *L. lactis* in, respectively, secretion of inactive pre-nisin and self-immunity to mature, active nisin. The 3D structures of PCAT1, of the same family as NukT, and McjD were adapted from PDB entries 4RY2 and 4PL0, respectively, using CCP4MG [87]. (b) Examples of antibiotic exporters of the MFS superfamily. Different shapes identify distinct entries in TCDB (Table 1). 'X^{+}' indicates that the identity of the ion gradient energizing transport has not been established or cannot be predicted for that particular transporter. ActA and ActB are shown paired, because they have been studied together, but their individual roles have not been established. Cmx and Ptr are included as respective examples of an AMR-linked exporter that originated from a BGC, and of an MDR efflux pump that is closely related to a BGC-linked exporter (here, VarS). All these examples are discussed in the text. CM/IM: cytoplasmic/inner membrane; PG: peptidoglycan; OM: outer membrane; TMD: transmembrane domain; NBD: nucleotide-binding domain; PEP: peptidase domain; RiPPs: ribosomally synthesised and post-translationally modified peptides.

Transporters – exporters and importers alike – are not only key players in innumerable biological processes, but they are emerging objects of interest in industrial biotechnology where their function can be exploited to optimize flux balances and enhance productivity in industrial strains [21]. Exporters are particularly attractive as means of detoxification, by which toxic end products can be relocated from inside the cell to the external milieu to give more durable and sustained outputs [22]. Importantly, even in the absence of toxicity, the ability to secrete the end product adds much value to the production process by reducing the costs and labour associated with product extraction and purification [21]. Therefore, we argue it is time for a new appreciation of transporter research in the context of antibiotic production.

Crucially, exporters also stand out for the direct role they play in AMR, if one only considers the important contribution of the so-called 'efflux pumps' during resistance in pathogens [23]. While long hypothesized, it has now been established that AMR-linked exporters can be phylogenetically related to exporters of antibiotic biosynthetic pathways, from which they have arisen through horizontal gene transfer (HGT) events [24]. This discovery expands to exporters the arsenal of AMR factors that natural antibiotic producers may disseminate in the environment and ultimately source to human pathogens [24, 25]. When the above observations are taken together, it becomes paramount that we understand how the function of different exporters is integrated within the wider process of antibiotic production, so that we can exploit its potential in antibiotic manufacturing and evaluate its risk for AMR dissemination.

Previous reviews by others have captured important aspects of antibiotic export, with some works focussing exclusively on bacterial ABC exporters [26, 27], other works touching on export while discussing self-immunity in producers [28] or the overall biology of specific antibiotic classes [29–31], and finally works dealing more generally with secondary metabolite exporters of different phylogenetic and organismal origins [15]. Drawing from all these examples, here we put together an updated compendium encompassing all families of bacterial exporters that nonetheless are specifically linked to antibiotic production (Fig. 1, Table 1). Our aim is to lay the foundations of a framework with which to connect the properties of antibiotic exporters in native producers to both their suitability for biotechnological applications and their likelihood to evolve in to AMR factors. Through a selection of significant examples, we first illustrate the many different ways BGC-linked exporters may be implicated with antibiotic production, and we finally discuss what impact this information might have on the needs of the biotech industry on one side and the concerns of AMR research on the other.

Reassessment of transporters linked to antibiotic export

The literature contains a large and disparate body of evidence that has linked genes encoding potential exporters to antibiotic production. For consideration in this review we used strict criteria for inclusion, which were as follows: (i) that the exporter genes be genetically linked to the BGC; (ii) that they code for at least one membrane protein component of an acknowledged exporter family; and (iii) that there be experimental evidence to implicate the action of these exporters with antibiotic production (Table 1). This was an important quality control due to many indirect assertions of roles in export that confuse the literature. For example, an important 'false positive' in this field are the ABCF proteins, which are now known to use their ATPase activity to function directly in ribosome protection [32] and not to form part of novel ABC efflux systems as had been hypothesized for a long time (for an excellent recent discussion of ABCF proteins see [33]). The audit also revealed significant differences in the experimental evidence used to study antibiotic export. Exporters have collectively been implicated in all aspects of antibiotic production, including, e.g. biosynthesis proper (i.e. the physical maturation of the antibiotic molecule), BGC transcriptional regulation, and self-immunity. This is compounded by the plethora of experimental approaches and techniques, *in vivo* and *in vitro*, genetic and biochemical, that researchers have used over the years to inform different questions as to the physiological roles, substrate specificity and mechanisms of export.

The compendium we produced is contained in Table 1, where it is clear that exporters belong to many different families and subfamilies of transporters, which we formalized by using TCDB identifiers [34] wherever possible, and come from organisms as diverse as Actinobacteria, Firmicutes and Proteobacteria. The following sections of the review examine examples of different evidence/functional types from this table to illustrate the variety of evidence and proposed functional roles of these exporters. This starts with exporters directly coupled to antibiotic biosynthesis/export and those with known roles in self-immunity.

Export directly linked to antibiotic maturation

Among the best-studied BGC-encoded transporters with strong evidence of a direct function in product export are examples where the exporter itself has a bifunctional role in the antibiotic's final maturation as well as export (Table 1, Fig. 1a). These examples are exporters of some RiPP (ribosomally synthesised and post-translationally modified peptide) antibiotics, namely class I and II lantibiotics, and a few of these have been described to molecular detail using *in vitro* assays with purified components, and crystal structures in some cases (Fig. 1a; see below).

For both classes of lantibiotics, the function of a dedicated ABC transporter, generally termed 'LanT' [31], is required for the actual formation of the final product. Structurally, ABC transporters are multisubunit complexes that use the direct binding and hydrolysis of ATP to drive the transport cycle, and usually consist of two integral membrane protein subunits and two ATPases.

Table 1. Exporters of antibiotic-producing bacteria

Exporter	SF[a]	TCDB	BGC[b]	Class	Producer	GO Evidence Codes[c]
NisT	ABC	3.A.1.111.3	Nisin	RiPP (Class I lantibiotic)	*Lactococcus lactis*	IDA[35], IMP 35, 36]
NisFEG	ABC	3.A.1.124.1	Nisin	RiPP (Class I lantibiotic)	*Lactococcus lactis*	IDA[88]
NukT	ABC	3.A.1.111.7	Nukacin IKS-1	RiPP (Class II lantibiotic)	*Staphylococcus warneri* ISK-1[d]	IDA[37–39], IMP [38]
PCAT1	ABC	3.A.1.112.9	Cthe_0535[e]	RiPP	*Clostridium thermocellum*	IDA[40]
McjD	ABC	3.A.1.118.1	MccJ25	RiPP (lassopeptide)	*Escherichia coli*[d]	IDA[44, 45], IMP [43, 46]
McbEF	ABC	3.A.1.116.1	MccB17	RiPP (microcin)	*Escherichia coli*[d]/ *Pseudomonas syringae*	IMP[89, 90]
KlpE	ABC		Klebsazolicin	RiPP	*Klebsiella pneumoniae* subps. *ozaenae*	IDA[91][f]
CinTH	ABC		Cinnamycin	RiPP	*Streptomyces cinnamoneus* DSM 40646	IMP[92]
MibEF	ABC		Microbisporicin	RiPP	*Microbispora corallina*	IMP[70]
PspEF	ABC		Planosporicin	RiPP	*Planomonospora alba*	IDA[69][f], IMP[67]
PspTU	ABC		Planosporicin	RiPP	*Planomonospora alba*	IMP[69]PspYZ
	ABC	3.A.1.148.2	Planosporicin	RiPP	*Planomonospora alba*	IMP[69]
Tba	ABC		Balhimycin	Glycopeptide	*Amycolatopsis bahlimycina*	IDA[93]
MoeX5-P5	ABC		Moenomycin	Phosphoglycolipid	*Streptomyces ghanaensis* ATCC14672	IDA[79]
CmrAB	ABC	3.A.1.105.3	Chromomycin	Aureolic acid	*Streptomyces griseus* subsp. *griseus*	IDA[55][f]
MtrAB	ABC		Mithramycin	Aureolic acid	*Streptomyces argillaceus*	IDA[f], IMP[56]
AvtAB	ABC		Avermectin	Macrocyclic lactone	*Streptomyces avermitilis*	IDA[74]
PpzR1, PpZR2[g]	ABC		Endophenazines	Pyrazine	*Streptomyces anulatus* 9663	IGI[94]
DrrAB	ABC	3.A.1.105.1	Doxorubicin	Anthracycline	*Streptomyces peucetius*	IDA[51, 52][f], IMP [53]
NysGH[h]	ABC		Nystatin	Polyene macrolide	*Streptomyces noursei* ATCC 11455	IMP[42]
OleC4-C5	ABC	3.A.1.105.2	Olenadomycin	Macrolide	*Streptomyces antibioticus*	IDA[95, 96][f]
PltHIJKN	ABC	3.A.1.105.4	Pyoluteorin	NRPS-PKS hybrid[i]	*Pseudomonas* spp.[j]	IDA(97)[f], IMP (97, 98)
NovA	ABC		Novobiocin	Aminocoumarin	*Streptomyces niveus*	IDA[99][f], IGI[100]
SimX	MFS		Simocyclinone	Aminocoumarin	*Streptomyces antibioticus* Tü 6040	IDA[72][f]
CouR5	MFS		Coumermycin A_1	Aminocoumarin	*Streptomyces rishiriensis* DSM 40489	IDA[99][f]
MccC	MFS	2.A.1.61.1	MccC51	RiPP (microcin)	*Escherichia coli*[d]	IMP[101]
BotT	MFS		Bottromycin	RiPP	*Streptomyces* sp. BC16019	IMP[73]
TblR	MFS		Tabtoxin	β-lactam	*Pseudomonas syringae* BR2	IGI[102]
LanJ	MFS	2.A.1.3.32	Landomycin A	Angucycline	*Streptomyces cyanogenus* S136	IDA, IMP[103]
LndJ	MFS	2.A.1.3.32[k]	Landomycin E	Angucycline	*Streptomyces globisporus*	IDA, IMP[75]
AbmD	MFS		Abyssomycin	Tetronate	*Streptomyces koyangensis* SCSIO 5802	IMP[104]
VarS	MFS	2.A.1.3.5[l]	Virginiamycin S	Streptogramin B	*Streptomyces virginiae*	IDA[62, 105][f]
PhlE	MFS	2.A.1.45.1	2,4-diacetylphloroglucinol	Phenolic compound	*Pseudomonas fluorescenes* F113	IMP[106]
Hyg19	MFS		Hygromycin A	Aminoglycoside	*Streptomyces hygriscopicus* NRRL 2388	IMP[107]
TcmA	MFS	2.A.1.3.12	Tetracenomycin	Anthracycline	*Streptomyces glaucescens*	IDA[108][f]
Mmr	MFS		Methylenomycin	Cyclopentenoid	*Streptomyces coelicolor* A3(2)[d]	IDA[109, 110][f]
FacT	MFS		Factumycin	Elfamycin	*Streptomyces* sp. WAC5292	IDA[111][f]
OtrB	MFS	2.A.1.3.29	Oxytetracycline	Naphthacene	*Streptomyces rimosus*	IDA[57–59][f], IMP[60, 61]
LmrA	MFS	2.A.1.3.9	Lincomycin	Lincosamide	*Streptomyces lincolnensis*	IDA[112, 113][f]
Mct	MFS		Mitomycin C	Aziridine-	*Streptomyces lavendulae* NRRL	IDA[114, 115][f]

Table 1. cont.

Exporter	SF[a]	TCDB	BGC[b]	Class	Producer	GO Evidence Codes[c]
PurT (Pur8)	MFS	2.A.1.3.11	Puromycin	containing Aminoacyl-nucleoside	2564 *Streptomyces alboniger*	IDA[116, 117][f]
GouM	MFS		Gougerotin	Peptidyl nucleoside	*Streptomyces graminearus*	IDA[71], IMP[71], IGI[118]
ActC	MFS	2.A.1.3.7	Actinorhodin	BIQ[n]	*Streptomyces coelicolor* A3(2)	IMP[66]
ActA[m]	MFS	2.A.1.3.25	Actinorhodin	BIQ[n]	*Streptomyces coelicolor* A3(2)	IMP[63, 66]
ActB[m]	RND	2.A.6.5.1	Actinorhodin	BIQ[n]	*Streptomyces coelicolor*A3(2)	IMP[63, 66]
Examples of proteins originally assigned as BGC-linked exporters						
OtrC4-C5	ABC		MDR		*Streptomyces rimosus*	IDA[48, 57][f]
Ptr	MFS	2.A.1.3.5	MDR		*Streptomyces pristinaespiralis*	IDA[78][f]
OleB	ABCF	3.A.1.120.3	Oleandomycin	Macrolide	*Streptomyces antibioticus*	IDA[95][f]

a, superfamily

b, we use here the final product's name as a shorthand for each BGC. The final product, however, may not be the actual substrate of the exporter in each case.

c, Gene Ontology Evidence Codes. Based on the full definition of each GO evidence code (http://geneontology.org/docs/guide-go-evidence-codes/), we established that we only needed to use three codes, namely IDA (inferred from direct assay), IMP (inferred from mutant phenotype), and IGI (inferred from genetic interaction). We used the IDA evidence code whenever biochemical or cell biological assays were carried out to provide evidence for a transporter gene's molecular function and/or role in a biological process. We also used IDA whenever an expression system was used as a way to investigate the normal function of the gene. We used the IMP evidence code in those cases when the function or biological role of a transporter gene was inferred based on differences in behaviour between two different alleles of that gene, e.g. in comparisons between WT v. deleted/KO loci. We also used IMP when heterologous expression was used to compare alleles. Finally, we used IGI for studies where transport genes were deleted or manipulated together with other genes in the BGC, and for functional complementation studies where a transporter gene from one organism was used to functionally replace that of the native organism. Numbers in parenthesis behind GO evidence codes identify the literature references that we used to understand if and how each transporter was implicated in BGC function.

d, plasmid-borne BGC.

e, this BGC is largely uncharacterized.

f, confers resistance upon heterologous or ectopic expression.

g, the genes for these exporters were not studied individually, but within a polycistronic fragment containing also other genes. Each protein is a TMD+NBD fusion.

h, while the evidence suggests NysG and NysH form a heterodimer, this is not definitively demonstrated and the two transporters could function independently.

i, NRPK: non-ribosomal peptide synthetase; PKS: polyketide synthase.

j, this envelope-spanning complex has been studied in both *Pseudomonas* sp. M18 and *P. protegens* Pf-5, but using different gene names. Fig. 1 uses the nomenclature of [97], also adopted by TCDB.

k, based on>70 % ID with LanJ.

l, based on>70 % ID with Ptr.

m, in the reference provided, the *actAB* genes have been studied as a bicistron, not individually – see the main text.

n, benzoisochromanequinone.

These subunits may be encoded by any combination of separate genes or domain fusions (Fig. 1a) [26], but in the case of LanT exporters all domains are usually fused into a single polypeptide (Fig. 1a). Class I and II lantibiotic exporters use different solutions to link RiPP maturation to export. We illustrate these two strategies using examples of the exporters for (pre-)nisin (class I) and nukacin (class II). Due to the high degree of conservation of biosynthetic logic among lantibiotics [27, 31], we do not include in Table 1 all other instances of class I and II lantibiotic exporters, examples of which can be found in [27].

The nisin system from *Lactococcus lactis* uses the ABC transporter NisT [35, 36] (Fig. 1a). Like all RiPPs, nisin is synthesized from the ribosome as a prepropeptide, NisA, containing a leader sequence that directs the post-translational modification of NisA's core and is eventually removed. Unlike most RiPPs, however, class I lantibiotics are secreted in an inactive form that still carries the leader peptide, and are then activated by the extracellular protease NisP [27, 31]. NisT is required for the secretion of pre-nisin, and uses the leader peptide for substrate recognition [35, 36]. In heterologous expression studies, van den Berg van Saparoea and colleagues [36] showed that in the

absence of NisT the amount of extracellular pre-nisin (NisP was not included in the reconstructed system) dropped 100-fold, and a probable nisin degradation product concomitantly accumulated in the cytoplasm. The class I lantibiotic exporter NisT then serves as a crucial player in nisin production in *L. lactis* by bridging the biosynthesis of pre-nisin in the cytoplasm with nisin's activation on the outside.

The biosynthesis of class II lantibiotics fully integrates the exporter into the maturation process, as exemplified by the case of nukacin ISK-1 from *Staphylococcus warneri* ISK-1 [37]. In this case, the ABC exporter NukT, which now incorporates a dedicated peptidase domain (PEP) at its N-terminus, is directly responsible for removing the leader peptide from the RiPP precursor, which has been shown both *in vivo* and *in vitro* [37–39] (Fig. 1a). Leader peptide cleavage by NukT occurs in the cytoplasm before secretion on the fully post-translationally modified substrate, and it is required for substrate recognition and export by NukT [38]. Using both mutagenesis studies of NukT heterologously expressed in different hosts and elegant *in vitro* assays with NukT reconstituted in proteoliposomes, the Sonomoto group in Japan established not only that the PEP domain plays an essential role in export, but also that PEP and NBD domains act cooperatively stimulating each other's activity [38, 39].

Because of their structural and functional properties, NukT-like ABC transporters are referred to as either AMS (ABC transporter for maturation and secretion) [38] or PCAT (peptidase-containing ABC transporter) [40]. The cooperation between PEP and NBD domains within NukT represents a remarkable device to subordinate export to the maturation process, and insight into how this is achieved is offered by the crystal structure of the related PCAT1 transporter from *Clostridium thermocellum* (Fig. 1a), which shows extensive contacts between the PEP domain and the transporter core, including the NBDs [40]. While the *C. thermocellum* system has not been investigated *in vivo* so far, the *in vitro* characterization of PCAT1's interaction with the putative substrate, the bacteriocin-like *Cthe_0535* gene product, established the PEP domain as the docking site for the substrate on the transporter, and also demonstrated that optimal cleavage is only achieved through a physical interaction between PEP domain and transporter core [40]. Intriguingly, the Sonomoto group [39] has raised the point that there might be differences in the way PEP–NBD coupling works in different PCAT/AMS exporters: whilst the PEP and NBD domains of PCAT1, expressed and purified separately, still appeared to cooperate (at least to a degree), those of NukT did not, indicating that the full-length architecture of the protein mediates coordination in this case [39, 40]. In this context, noteworthy are some results in [38], where a control mutant of NukT, carrying a single mutation in the PEP domain external to the catalytic site, displayed increased peptidase activity in membrane vesicles, and led to a 50 % increase in the amount of mature nukacin *in vivo*. It is not established whether this mutant truly reflected some altering to PEP–NBD coordination, but its phenotype tantalizingly suggests there might be further layers of regulation underpinning NukT's function.

Importantly neither of these NisT-like and NukT-like exporters are involved in resistance to the active (extracellular) form of the lantibiotic, which instead relies on the synergistic action of a different ABC exporter, generically termed LanFEG, and of an accessory membrane-associated protein (LanI or LanH), all encoded within the same cluster as *lanT* [27] (Fig. 1a illustrates the case of NisFEG and NisI). While LanFEG exporters are not ubiquitous among lantibiotic producers, and their impact to resistance may vary [41], their mechanism of action may involve extraction of exogenous lantibiotic embedding itself into the cell membrane, followed by handover to LanI/LanH for final removal [41]. LanT- and LanFEG-type exporters, whilst both ABC transporters, are not closely phylogenetically related [27].

Transporters may affect product maturation in other ways. An intriguing example is provided by the ABC transporters NysG and NysH (Fig. 1a) involved in the biosynthetic export of the antifungal macrolide nystatin in *Streptomyces noursei* [42]. Deletion of either transporter gene caused a ca. 35 % reduction in the levels of fully formed nystatin and led to concomitant enrichment of a deoxy precursor. Plasmid-borne copies of either gene could only complement their cognate deletion, indicating that NysG and NysH work in concert [42] or might even form a heterodimer (as tentatively depicted in Fig. 1a). Over-expression of the hydroxylase NysL could partly alleviate the phenotype of the transporter mutants [42]. Remarkably, nystatin, either in its mature form or as its deoxy precursor, appeared to be normally secreted regardless of *nysG* and *nysH* deletions, indicating that other transport systems contribute to export in the producer [42]. To explain these data, the authors hypothesized that a NysGH complex might preferentially export fully formed nystatin and that this activity would increase the efficiency of the last biosynthetic step through product removal [42]. In the absence of NysGH, biosynthetic efficiency would be compromised, but other, less selective transport systems would take over secretion of both nystatin and the deoxy precursor [42]. Further investigation is needed to clarify the function and specificity of NysGH as a nystatin exporter, not to mention the interplay with other possible transporters, but this work may be taken to illustrate the important point that the ability to extrude the final product might affect flux balances and have an impact on product maturation.

Effect on self-immunity

Some transporters play a crucial role in preventing self-intoxication of an antibiotic producer by pumping out the final, active product before it reaches toxic levels in the cytoplasm. If detoxification is somehow compromised, production may be negatively affected, and cell viability may also drop drastically.

The clearest example of an exporter coupled to biosynthesis to prevent self-intoxication is provided by the McjD ABC exporter (Fig. 1a) from the BGC of the lassopeptide Mccj25 (another RiPP), produced by certain plasmid-bearing *E. coli* strains [43–45]. Disruption of *mcjD* was lethal to cells that carried the rest of the plasmid-borne cluster [43, 46]. The lethality of the *mcjD* deletion could be overcome by a spontaneous mutation in the intracellular target, with these mutant cells accumulating mature Mccj25 in the cytoplasm [43]. Expression of *mcjD* in these cells restored secretion of Mccj25 into the external milieu [43]. Interestingly, expression of *mcjD* could confer resistance to exogenously added MccJ25 in *E. coli* [46], which raises questions as to how the substrate enters McjD.

McjD has been characterized at atomic and mechanistic level by a series of elegant studies led by the Beis group in London in collaboration with other laboratories [44, 45, 47] (Fig. 1a). High-resolution crystal structures of McjD showed an occluded conformation that atypically remained so in either apo- or ADP-VO_4-bound forms [44, 45]. PELDOR measurements confirmed these data, while evidence for transient opening was provided by Hoechst transport assays and cross-linking experiments in the presence of Mccj25 [44]. The authors commented that this semi-permanent occlusion of the internal, putative substrate-binding cavity within the transporter might be at the bases of the transporter specificity for MccJ25, thus constituting a key difference from MDR exporters, which tend instead to display more open structures 'at rest' [44]. Very recent *in vitro* studies using solution NMR and ATPase assays have investigated the specificity of McjD towards MccJ25, and identified a stretch of four residues within the substrate as the primary recognition site for export [47]. This is important knowledge that can inform rational design of substrate variants of pharmaceutical interest that can still be exported by McjD, and we await further studies on this very interesting system.

Indirect evidence (effect on resistance in heterologous hosts)

Roles in self-immunity have also been historically assigned to those transporter genes that, when heterologously expressed, could confer resistance to exogenously added antibiotics. This strategy to identify dedicated antibiotic exporters is a major source of 'false positives', which besides the blatant case of the ABCF proteins mentioned above also include transporter genes not linked to a BGC. For instance, genuine MDR efflux pumps like OtrC4-C5 (Table 1; 'OtrC' in much of the literature) and Ptr (Table 1, Fig. 1b), have been assigned roles in native antibiotic production, because they could confer heterologous resistance to various molecules including the target, but, with their genes not residing within the relevant BGCs and being regulated independently [48–50], it is not clear how, if at all, their MDR qualities are attuned to BGC functions. While we can still make important points from these examples (see below), we think these proteins should not be ranked as true BGC-linked antibiotic exporters until more evidence becomes available.

The use of heterologous expression must be understood as a tool to discover and start investigating uncharacterized exporter genes, while providing indirect evidence for a native role in self-immunity. Unlike other types of genetic studies, heterologous expression has the immediate positive outcome that it starts informing us on the substrate specificity of the exporters. For some of these exporters there is accompanying direct evidence that they play a native role in self-immunity; however, whether this holds true for the entire group is open to investigation. We present here some significant examples to illustrate this diverse experimental landscape.

The daunorubicin/doxorubicin ABC exporter DrrAB from *Streptomyces peucetius* (Fig. 1a) was originally assigned as a resistance factor through heterologous expression in *S. lividans* [51] and in *E. coli* [52]. It was a later work [53], however, that helped clarify the physiological role of the transporter in the producer. After making a double *drrAB* mutant, which proved more sensitive to exogenous dauno-/doxorubicin and also produced less antibiotic, Srinivan and colleagues [53] connected the observed phenotype to a feedback transcriptional regulatory mechanism, where deficient export has a part in downregulating some of the biosynthetic genes (we will see more examples of regulatory roles below). The authors reported they could not produce a triple deletion mutant lacking *drrAB* and *drrC* [53], where DrrC is UvrA-like additional resistance factor [54]. Since individual *drrAB* and *drrC* deletions could be made in *S. peucetius*, the authors argued for a cooperative role of DrrAB and DrrC in self-immunity [53].

Heterologous expression studies of the ABC exporter, CmrAB, encoded within the chromomycin A3 BGC of *S. griseus*, led the authors to important insights as to the transporter's substrate specificity and, combined with more evidence, to the possible physiological role in the natural producer [55]. Compared to the entire BGC, the *cmrAB* genes could only confer partial resistance to chromomycin A3 in the heterologous host *S. albus* and needed the synergistic action of the *uvrA*-like gene, *cmrX* for the full effect [55]. The *cmrAB* genes were however sufficient for full resistance against the closely related antibiotic mithramycin, and, significantly, also against the di-deacytaled precursor of chromomycin A3, DDACA3, which has lower yet detectable antibiotic activity [55]. Chromomycin A3 is produced from DDACA3 by the action of the membrane-bound acetyltransferase CmmA, and, consistently, a *cmmA* deletion mutant of *S. griseus* could only make DDACA3 [55]. As DDACA3 was still released into the supernatant, the authors concluded that this precursor might be the true substrate of CmrAB [55]. A prominent role for CmrAB in self-immunity to DDACA3 was hypothesized following failed attempts to delete *cmrAB* in *S. griseus*, especially when considered that adjacent genes including *cmrX* could be deleted [55].

It is worth noting that, while the specificity of CmrAB seems to be partly relaxed, that of the equivalent ABC transporter from the mithramycin BGC, MtrAB from *S. argillaceus*, does not, as heterologous resistance was only seen to mithramycin [56]. Mithramycin and chromomycin A3 share the same aglycone structure, but they differ by the type of glycosylation and accompanying acetylation that subsequently modify this core [55]. We do not know what the molecular bases might be for this difference in substrate specificity, and only comparative structure-function studies on CmrAB and MtrAB can start informing this very interesting question.

Heterologous expression was also used to identify oxytetracycline-resistance factors from the industrially relevant natural producer, *Streptomyces rimosus* [57], of which one was the transporter, OtrB (Fig. 1b). OtrB is a member of the superfamily of MFS transporters, which unlike ABC transporters are energized by proton or cation gradients and are normally encoded by a single gene (Fig. 1b). The *otrB* gene has been independently isolated twice (under different names) by transforming sensitive organisms such as *S. griseus* and *S. lividans*, with genomic libraries from *S. rimosus* and *Kitasatospora* (formerly *Streptomyces*) *aureofaciens*, which were then selected for growth on tetracycline [58, 59]. *otrB*-expressing transformants were found to be resistant to as high as >200 μg ml^{-1} tetracycline [58].

Two more (oxy)tetracycline resistance factors from *S. rimosus* have been isolated through heterologous expression: OtrA, an elongation factor involved in ribosome protection, and the ABC transporter mentioned above, OtrC4-C5 ('OtrC'), responsible for resistance to a range of antimicrobials including tetracycline (47, 56; see also references therein). The interplay among these three factors in determining specific resistance to oxytetracycline, and most importantly in actively producing cells, has not been investigated, nor has the exact physiological role of OtrB in *S. rimosus* [57], although some works showed that overexpressing *otrB* in the producer may lead to a modest increase in oxytetracycline production [60, 61]. Whatever this native role might be, we should remark that the *otrB* gene has been shown to be a substrate of HGT leading to AMR (24; see below). The case of OtrC4-C5 informs us as to how more information is really required to assign a BGC-linked transporter: the *otrC* locus was found to confer only low and unspecific resistance to tetracycline in heterologous hosts, and it is not physically linked to the *otr* cluster in *S. rimosus*, indicating OtrC4-C5 might act as a 'generalist' MDR efflux protein rather than a dedicated oxytetracycline exporter [57]. This argues for OtrB to be the main route of oxytetracycline export during production [57].

Finally, another intriguing case is provided by the MFS transporter, VarS, implicated in virginiamycin export (Fig. 1b). Virginiamycin, produced by *Streptomyces virginiae*, is a streptogramin comprising of two separate antibiotic components, Virginiamycin M1 (VM1) and Virginiamycin S (VS), which are synthesized by a 'super cluster' and are most active when present as a mixture [50, 62]. VarS was confirmed to play a role in efflux after expression of its gene in *S. lividans* could confer resistance to both sole VS and a 7 : 3 mixture of VM1:VS [62]. As the intrinsic tolerance to VM1 in *S. lividans* was unaltered by *varS*, the authors concluded that VarS was specific to VS [62]. While the physiological role of VarS in *S. virginiae* has not been established, one should be cautious with the conclusion that VarS cannot export VM1. If resistance to given external concentrations of antibiotic is used as a proxy for export function, we must then point out that *S. lividans* without *varS* was already resistant to a concentration of VM1 much higher than that used for the VM1:VS mixture, and no other concentrations were tested [62]. The experimental conditions make it impossible to discern any VM1-specific effects, and we cannot rule out that VarS might be able to export both components. However, if we do espouse the hypothesis that VarS is specific for VS, this raises the interesting questions as to which export route is taken by VM1, and how the two export processes might be coordinated to ensure that the mixture – the most active form of the antibiotic, is delivered when required.

It is clear from the above examples that heterologous expression lets us into important features of antibiotic export and is a powerful tool to isolate new exporter genes. Our take-home message is ultimately that one should always appraise the available evidence very carefully before drawing conclusions as to how exporters identified in this manner relate to the functioning of the BGCs of interest.

Effect on regulation of BGC expression

The studies on DrrAB have introduced us to the concept that antibiotic exporters may play key roles in regulating the expression of their home BGCs. Well-studied and highly significant examples of this ability are ActA (ActII-ORF2) and ActB (ActII-ORF3), respectively MFS and RND (root-nodulation-division) exporters, involved in actinorhodin production in *Streptomyces coelicolor* (Fig. 1b). ActA and ActB are crucial players in the sophisticated feed-forward mechanism proposed by the Nodwell group in Canada, by which secretion of the end product by an actively producing subpopulation within a culture leads to the transcriptional activation of the BGC in the rest of the cells [63, 64]. The starting observation was that cultures of the double *actAB* mutant could only produce a fifth of the levels of actinorhodin compared to WT [63, 64]. This phenotype was explained using a set of very elegant genetic studies involving a mutant form of the ActR repressor: as first hypothesized by Tahlan and colleagues [64], Xu and colleagues [63] showed that activation of actinorhodin production occurs in two waves where expression of key *act* genes, including the *actAB* operon, is initially induced by a biosynthetic intermediate; this transient transcriptional burst ultimately results in actinorhodin production, but it grows into full induction only once the accumulating actinorhodin is secreted out of the cells. The results of cross-feeding

experiments mixing an actinorhodin-producing strain with isogenic non-producing reporter strains were consistent with extracellular actinorhodin serving as a signal secreted by an actively producing subpopulation to induce sustained activation of the pathway within the entire culture [63, 64].

Despite the undeniable links between *actAB* gene expression and actinorhodin production, which strongly suggest that actinorhodin might be indeed secreted *via* ActA and ActB, the authors recommended caution with identifying ActA and ActB as *bona fide* actinorhodin exporters as their export activity was not investigated directly, and some actinorhodin was still made and secreted in the double *actAB* mutant [63] (see also below). A role for ActA and ActB in preventing self-intoxication was also discussed, as a strain that could activate *actAB* only transiently showed stunted growth over longer fermentation times [63].

ActC (ActVA-ORF1) is a third exporter (MFS) that has been implicated in actinorhodin production based on its gene upregulation in response to pH drops, which is concomitant with enhanced actinorhodin secretion [65, 66]. While *actC* mutants have been made and phenotyped to a degree [66], the exact role of ActC in actinorhodin secretion/production and the interplay among the three potential exporters are largely unexplored, though the interesting possibility has been put forward by Xu and colleagues [63] that ActC might be responsible for the residual actinorhodin export in the double *actAB* mutant.

Feed-forward mechanisms may explain the regulation of BGCs for the actinobacterial RiPPs, planosporicin and microbisporicin [67, 68], studied by the Bibb group in Norwich, though the exact roles of the multiple ABC transporter genes in these clusters, which also seem to modulate production and self-immunity [69, 70], have not been elucidated. There is evidence for regulatory mechanisms, likely in the form of feedback inhibition, in other systems, namely the gougerotin BGC from *Streptomyces graminearus* expressing the GouM MFS transporter, and the simocyclinone BGC from *S. antibioticus* Tü 6040 expressing the SimX MFS transporter [71, 72], though in the latter case the effects on product yield were not investigated [72]. Export might also regulate the expression of the bottromycin gene cluster from *Streptomyces* sp. BC16019, as making the MFS transporter gene, *botT*, constitutive increased bottromycin production in a heterologous host [73].

Taken together, these works suggest that regulatory mechanisms might represent a recurrent device to ensure that the exporter function is fine-tuned with antibiotic production.

Exploitation of BGC-linked exporters in biotechnology

The function of specific antibiotic exporters can be harnessed for biotechnological purposes [21, 22], and we discuss here two examples where the effects of transporter manipulation have been investigated in industrial/over-producing strains.

The genes for the AvtAB ABC transporter map to and are co-expressed with the BGC for avermectin in *Streptomyces avermitilis* [74]. Avermectin targets parasites and the natural producer does not need a self-immunity mechanism, suggesting AvtAB might have a role in production [74]. In the WT strain, deletion of either *avtA* or *avtB* had no effect on avermectin production, while over-expression of *avtAB* lead to increased levels of all eight avermectin congeners with twofold increased levels for the B1a congener, the only one that could be quantified with accuracy [74]. The same phenotype could be reproduced in an industrial over-producer [74]. Increased production could be shown to be specific to avermectin in both the WT and industrial strains by investigating the levels of olygomycin A, another product made by *S. avermitilis*, which were found unaltered [74]. Strengthening the hypothesis that AvtAB function affected production through increased secretion, the ratio between extracellular and intracellular avermectin in the modified strains was found to be slightly, but significantly increased [74].

Landomycin E production by *Streptomyces globisporus* was also found to respond to over-expression of the BGC-linked transporter gene, *lndJ*, coding for an MFS transporter [75] (Fig. 1b). However, this study has important differences compared to that of *S. avermitilis*/avermectin, in that the responses of the WT strain and over-producing strain were very different [75], and the interpretation of their respective phenotypes is in our opinion not straightforward. While deletion of *lndJ* had no effect on either production or resistance in the WT [76], over-expression of *lndJ* in this same strain increased resistance to exogenous landomycin E, but also virtually abolished production [75]. However, in an over-producing strain the same *lndJ* over-expression strategy (*ermE**p control on a multi-copy plasmid) had only a small effect on resistance to exogenous product, while the effect on production was the appearance of a second landomycin form alongside control levels of landomycin E [75].

To try and rationalize this *S. globisporus*/landomycin E study, we can only speculate that the genetic adaptations giving the industrial strain its over-producing phenotype might lead to a very different physiological and/or regulatory environment for transporter function to fit in, thus causing a different response compared to WT.

Both studies bear the important biotechnological implication that transporter function can still be manipulated to advantage in industrial strains, even though these have already been evolved/engineered for increased production. Even small increases in yields can result in considerable improvements when fermentation is scaled up to industrial level. There is possibly additional insight to gain from the landomycin E study, in that this work makes a case for investigating the effects of transporter manipulation in improved strains regardless of what is observed in a reference WT strain.

It is worthwhile to point out that genetic and structure-function studies can also provide insights of biotechnological value, even when not directly addressing strain improvement. Think of the above examples of the hyperactive NukT 'control' mutant that led to increased nukacin yields in culture [38], and of the over-expressed *botT* gene that led to reactivation of the bottromycin BGC in a heterologous host [73]. It is reasonable to expect that, as research unveils functional and mechanistic properties of antibiotic exporters, this new knowledge might inform and expand the repertoire of approaches at our disposal for strain engineering.

Importance of BGC-linked exporters to AMR

Finally, we wish to consider the relationships that might exist between production-linked export and AMR. It is noteworthy that some BGC-linked exporters, such as, e.g. OtrB from *S. rimosus* (see above), have been identified because they could function as resistance factors in heterologous hosts. Even when a native role in self-immunity is not confirmed, their ability to function 'out of context' makes these proteins potential substrates of HGT, and in fact there is now evidence that some exporter genes, including *otrB*, have migrated from soil Actinobacteria to environmental and even pathogenic Proteobacteria through a so-called 'carry back' mechanism [24]. Using primarily the examples of *cmx* (Fig. 1b) and *lmrA*, encoding respectively chloramphenicol and lincomycin exporters, Jiang and colleagues [24] showed how 'carrier' sequences of proteobacterial origin can first use conjugation to migrate into actinobacterial antibiotic producers, acquire therein native self-immunity factors including exporter genes through mechanisms of genome plasticity (e.g. transposition), and finally return to Proteobacteria through natural transformation and homologous recombination. While the evidence primarily relies on very sophisticated phylogenetic studies, *in vivo* experiments crucially showed that *cmx* could be transferred from the soil actinobacterium, *Corynebacterium resistens*, to the Gram-negative pathogen, *Acinetobacter baylyi* [24]. We can thus add transporter genes to the list of environmental resistance factors that originate from BGCs [25]. As another example of genetically related yet functionally distinct groups, one should think of the LanFEG-type and the CprABC-type families of lantibiotic ABC exporters, the former highly specific and contributing to self-immunity in producers, and the latter of broader specificity and involved in AMR to RiPPs [41]. As bacterial genomes are being sequenced in ever-larger numbers, we anticipate further phylogenetic studies that will highlight many such cases of HGT of exporter genes and will clarify the mechanisms of transfer.

If BGC-linked exporters can spread *via* HGT, are there intrinsic features that make an exporter a likelier HGT substrate than another? And what are their phylogenetic relationships with the so-called MDR 'efflux pumps' [19, 77]? That evolutionary links might exist between these two groups has been discussed by different authors over the years [63, 75, 78]. As the most salient difference between BGC-linked exporters and MDR efflux proteins seems to be their substrate specificity, which can be very broad for members of the latter group, the question becomes one of how specificity evolves, and in which direction. In the absence of direct evidence, we are left to speculation, but one can imagine scenarios where a specific exporter leaves its native BGC to evolve MDR capacity (see below), and conversely where a BGC can recruit MDR efflux proteins for secretion. That heterologous expression of BGCs deleted for or naturally devoid of transporter genes still results in the final product's secretion [79, 80] lends credibility to the latter scenario. It has been discussed that MDR efflux proteins might even pre-date dedicated antibiotic exporters, and that capture plus adaptation to BGCs might have ultimately led to their specialization [78]. Phylogenetic studies on catabolic clusters indicate that transporter genes may be under selective pressure primarily for function, so that related clusters may carry transporter genes of completely different families that are nonetheless functionally interchangeable [81]. These studies concern substrate-specific importers, but one can see how the same logic may apply to antibiotic exporters, especially when these are MDR.

In the comparison between specific and 'generalist' exporters we can also see much scope for studies addressing the exact mechanism of transport, whereby we may gain insights into the molecular bases of substrate recognition and specificity. These are important questions that other research groups too have raised – for an example in the context of McjD specificity (see above; 46]). It is also worthwhile to point out that some class II lantibiotics exporters, such as EnkT, LahT and ProcT, have been shown or are predicted to transport multiple substrates [82–84], which makes them relevant subjects of study in this regard.

Studying exporters from producing organisms might give us insights into the links between MDR evolution and AMR dissemination. The noteworthy case of the MFS exporter, Ptr (Fig. 1b), unravelled through a number of publications, can be used to illustrate potential avenues of investigation. The pristinamycin super cluster from *Streptomyces pristinaespiralis* is highly syntenic to that of virginiamycin [50], which identifies the MFS exporter gene, *snbR*, as the orthologue of the candidate VS-exporter gene, *varS* (see above) [50], coding for a 75 % identical protein with a predicted similar function in pristinamycin export. Prior to these findings, this role had been assigned to *ptr* [50, 85], a gene isolated from a genomic library for the ability to confer pristinamycin and rifampicin resistance to heterologous hosts [78]. While the Ptr exporter is approximately 70 % identical to either VarS or SnbR, its gene lies in a mono-cistronic operon unlinked to the BGC, under independent and un-coordinated regulation [49, 85, 86]. In our view, a more accurate description of Ptr is as an MDR efflux pump, which was already suggested in [78]. Remarkably, *ptr* falls under the regulation of the heterologous transcriptional repressor Pip when expressed in non-native hosts [49, 86], which is due to conserved promoter sequences between *ptr*

and *pep*, Pip's native target, also coding for a highly similar exporter [86]. In its native hosts, Pep contributes to MDR against pristinamycin and other antibiotics, with Pip itself responding to various exogenous molecules [86]. Others have already commented that there may be evolutionary connections among these closely related transporters [86]; our remark is that collectively these studies give us glimpses as to how substrate specificity might change through gene duplication followed by diversification, and how these resistance genes can spread outwards to new hosts. Rigorous phylogenetic studies on this family of transporters, attractive because of conserved members differing by physiological role and genetic context, might reveal important bases for the rise of MDR and the spread of AMR.

Conclusion

Through the above examples we gave a broad yet representative overview of bacterial antibiotic exporters from natural producers. While exporters belong to different protein families (Fig. 1, Table 1), the collective experimental evidence for roles in antibiotic production is also varied in nature, counting both genetic and biochemical studies, and it identifies different roles such as product maturation, gene expression regulation, and self-immunity, to mention just the most prominent ones (Table 1). If the literature is a treasure trove of information on exporters as an ensemble, at the level of individual exporters our knowledge is however far from complete, as some of the cases we presented above illustrate. Exporters make undoubtedly interesting subjects of study – physiologically and mechanistically, and the understanding of their contributions to antibiotic production can have a significant impact on different lines of inquiry.

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Conflicts of interest
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