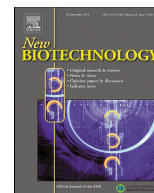
New BIOTECHNOLOGY 54 (2020) 13–27

Contents lists available at ScienceDirect

New BIOTECHNOLOGY

journal homepage: www.elsevier.com/locate/nbt



Review Article

Biodegradation of antibiotics: The new resistance determinants – part II

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ARTICLE INFO

Keywords:
Antibiotics
Transformation
Biodegradation
Subsistence
B-lactams
Macrolides
Quinolones
Ionophore antibiotics

ABSTRACT

Antibiotic residues are widespread in the environment and their presence is known to contribute to the propagation of antibiotic resistance. Nevertheless, knowledge on processes involved in their degradation is scattered. This second part of a two part review aims at compiling knowledge on the (bio-) degradation of antibiotics, focusing on β-lactams, macrolides, quinolones and ionophores, as well as some less common classes. Detailed metabolic and molecular aspects are discussed, as well as the role of antibiotic degraders in natural microbial communities. This exercise led to the conclusion that among the classes analyzed, the majority of antibiotics are prone to microbial cleavage or transformation.

Introduction

In the first review article, biodegradation of sulfonamides, trimethoprim, aminoglycosides, amphenicols, and tetracyclines was examined [1]. This review focuses on β-lactams, macrolides, quinolones, ionophore antibiotics and other minor classes of antibiotics, namely, oxazolidinones, nitroimidazoles, glycopeptides, lincosamides, lipopeptides and quinoxaline-*N,N'*-dioxides. As in [1], their chemical properties and function are examined by class, the main abiotic pathways of degradation in environmental systems are summarized and thereafter biodegradation by complex microbial communities, axenic microbial cultures and specific enzymes are discussed.

β-Lactams

β-Lactams (see suppl. Tables S1 and S2 for detailed information on physicochemical properties) are inhibitors of cell wall synthesis in bacteria, with bactericidal properties [2]. Currently, resistance to these antibiotics is highly prevalent, occurring mainly via hydrolysis of the β-lactam ring by β-lactamases and by mutated forms of penicillin-binding proteins [2]. Thousands of β-lactamases have been described and a comprehensive database is actively maintained by the National Center for Biotechnology Information (NCBI) [3]. Nevertheless, due to their broad-spectrum and low toxicity, β-lactams remain one of the most important groups of antibiotics both in human and veterinary therapy [4–6]. Detailed reviews on the different sub-classes and generations of β-lactam antibiotics can be found elsewhere [7,8].

Abiotic degradation

Despite their intensive use, these compounds are infrequently detected in environmental samples [9,10]. Low contamination levels may result from the inherent instability of the β-lactam ring, which is readily hydrolyzed both abiotic and enzymatically [11–13]. Hydrolyzed β-lactams (AMX-a, suppl. Figure S1) lack antimicrobial activity; conversely, they appear to be more recalcitrant than the original drugs [14] as they have frequently been detected in significantly higher concentrations in both environmental samples and animal tissues [15–18].

Beside hydrolysis, β-lactams are also susceptible to organic matter-mediated photolysis [19]. However, these processes alone are insufficient to eliminate all antibacterial activity and to mineralize these drugs (suppl, Figure S1) [19,20]. For cephalosporins, photodegradation led to by-products that were more toxic and photo-stable than the parent drugs [21]. Photocatalytic methods, such as TiO_2-photocatalysis or photo-Fenton processes can eliminate β-lactams [22–26], but to ensure reasonable breakdown, extended irradiation times are required ($\geq$ 10 h) [23,26] and the toxicity assessment of the possible metabolic products is often neglected. In fact, the application of strong oxidative

Abbreviations: AAC, aminoglycoside acetyltransferase; CBT, closed bottle test; NCBI, National Center for Biotechnology Information; WWTP, wastewater treatment plant; ZWT, Zahn-Wellens Test

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https://doi.org/10.1016/j.nbt.2019.08.003
Accepted 11 August 2019
Available online 13 August 2019
1871-6784/ © 2019 Published by Elsevier B.V.

treatments, such as O_3/H_2O_2, was reported to decrease the biodegradability of penicillin-contaminated wastewaters due to the higher toxicity of the resulting by-products [27]. Given the general tendency of these methods to form toxic by-products, their application for the elimination of β-lactams in water should be carefully considered.

Biotransformation and biodegradation

Specific hydrolases able to cleave the β-lactam ring were found shortly after the discovery of these antibiotics. In fact, the first reported β-lactamase, AmpC, was described one year before the introduction of penicillin into clinical use [28]. These enzymes date back to ancient times, as microorganisms isolated from a pristine 4-million year old cave were able to hydrolyze both penicillins and cephalosporins [29]. More than 1300 unique variants of β-lactamases have now been described [28]. All variants hydrolyze β-lactams irreversibly into their corresponding penicilloic acids, which are devoid of antimicrobial activity, as reported for photolysis (AMX-a, suppl. Figure S1). Several attempts have been made to classify this ever-growing group of enzymes, and currently, two main classification schemes are generally accepted: the Bush-Jacoby-Medeiros classification [30–32] and the Ambler classification [33]. The first classifies the enzymes according to their substrate range and susceptibility to inhibitors (four main classes and multiple subclasses) while the second one groups them according to protein homology (classes A to D). The most relevant and alarming types of β-lactamase are: (i) the extended-spectrum β-lactamases (ESBLs) that hydrolyze most penicillins and cephalosporins [34] and belong to class A from the Ambler classification scheme, and (ii) the carbapenemases, also known as the versatile β-lactamases [35], that possess activity against all sub-classes of β-lactam antibiotics [28] and include enzymes from class A, B, and D.

Beyond these diverse hydrolytic enzymes, knowledge on alternative pathways for biotransformation and biodegradation of these drugs is relatively scarce. In fact, most studies have reported significant mineralization only for penicillin G in the Zahn-Wellens test (ZW) [36], in the closed bottle test (CBT) [37,38] and with ^{14}C-labelled penicillin [39]. In all the previous tests, the degradability of the antibiotics is measured in mineral medium with the test substrate as the single carbon source. However, ZW and CBT differ in their ultimate purpose: ZW measures degradability of recalcitrant compounds and uses a high amount of activated sludge as inoculum [40], whereas CBT measures ready degradability and uses a very low amount of activated sludge [41].

These results suggest that penicillin is readily degradable by activated sludge communities, independently from the origin and amount of inoculum used. Nevertheless, they should also be interpreted with caution since none of these studies assessed the metabolic pathway or identified the microorganisms involved in this process. Although not reporting the extent of mineralization, it was shown that drugs of the cephalosporins sub-class can also be biodegraded in tests with lake sediments [42]. Indeed, two *Pseudomonas* sp. strains [43] isolated from activated sludge have been found to degrade the cephalosporin cephalexin with the accumulation of 2-hydroxy-3-phenyl pyrazine (CEP-1, suppl. Figure S2), a product also detected after acid hydrolysis of other β-lactams including ampicillin [44] and cefaclor [45]. Studies under anaerobic conditions report a similar behavior for these drugs, with rapid degradation [46] and limited mineralization of the parent molecule [47]. In contrast, high-throughput studies investigating a β-lactam subsistence phenotype have suggested that this trait is particularly abundant both in soils [48], in several *Salmonella* sp. isolates from environmental and clinical samples [49] as well as in the strain *Klebsiella pneumoniae* Z1 [50].

Nevertheless, only two reports linked this phenotype to actual degradation of penicillin G [48,50]. Fig. 1 presents several biodegradation products identified [50], such as penicilloic acid (BET-1), penilloic acid (BET-2) and penillic acid (PEN-1) among others. These products were accumulated only transiently and further degraded into unknown metabolites. It was shown [48] that penicillin was first hydrolyzed to penicilloic acid (BET-1, Fig. 1) and further decarboxylated to penilloic acid (BET-2, Fig. 1). This pathway has already been described for abiotic transformation of these drugs (AMX-a and b, suppl. Figure S1). Indeed, in a study carried out with amoxicillin, mineralization appeared to result from a combination of abiotic and biotic processes, as the drug was first hydrolyzed abiotically to amoxicilloic acid (BET-1, Fig. 1) and further degraded through unknown mechanisms by the microbiota of the activated sludge [51]. Analyses of several soil isolates has indicated that soil microbiota are able to use benzylpenicillin (penicillin G, Fig. 1, R = benzyl)) as a carbon source [52]. In these four isolates of the *Burkholderia*, *Pseudomonas* and *Pandoreae* genera, genes arranged in a penicillin utilization operon (*put*) were identified that allow the breakdown of penicillin G via β-lactamases to benzylpenicilloic acid (BET-1, Fig. 1, R = benzyl) and via amidases to phenylacetic acid (PEN-4) that could apparently be channeled into the phenylacetic acid pathway to the central metabolism. All isolates exhibited growth on penicillin G. The subsistence mechanism was further demonstrated by transformation of the *put* operon into *E. coli*, enabling the transformant to grow on penicillin or benzylpenicilloic acid as a sole carbon source [52]. This study is also the first proof of a direct connection between a biodegradation and a resistance mechanism. Besides β-lactamases, other enzymes with acylase and esterase activity were found to degrade penicillin antibiotics [53], e.g. penicillin acylase (EC 3.5.1.11). This enzyme was initially found in *Penicillium chrysogenum* Q 176 [54] and subsequently reported for many other organisms, namely bacteria, yeast, and filamentous fungi [55]. It can cleave the β-lactam molecule into phenylacetic acid and 6-aminopenicillanic acid (PEN-4 and PEN-4.1, respectively, Fig. 1), making it invaluable for industry since PEN-4.1 can be used as a precursor in the synthesis of semisynthetic β-lactam antibiotics [56]. Furthermore, membrane-bound D-alanine carboxypeptidase from *Geobacillus stearothermophilus* (formally *Bacillus stearothermophilus*) [57], originally involved in cell wall maturation, was also shown to degrade penicillin G yielding phenylacetylglycine (PEN-5, Fig. 1). A similar pathway was observed with extracellular DD-carboxypeptidase-transpeptidase of *Streptomyces* sp. R61 [58], which degrades penicillin with the stoichiometric release of phenylacetylglycine and *N*-formyl-D-penicillamine (PEN-5.1, Fig. 1), albeit at a very slow rate.

A novel Baeyer-Villiger Monooxygenase (BVMO, GenBank accession no. GG705134.1) from an *Acinetobacter radioresistens* strain has been found to oxidize the β-lactam ring of imipenem (IMI-1, suppl. Figure S2) [59]. When overexpressed in *E. coli* this enzyme provided increased resistance to imipenem indicating that this gene by itself confers the resistance phenotype to its host. The reaction catalyzed by this versatile enzyme and depicted in suppl. Figure S2 could represent a novel method of degrading β-lactams. More studies are required to understand how widespread and relevant this transformation is regarding further degradability/assimilation of this antibiotic.

Some studies have attempted to elucidate the link between degradation and resistance to β-lactams. For instance, it was shown [60] that in the presence of ampicillin, β-lactamase producers were able to protect susceptible bacteria and to prevent the development of resistance in these strains, independently of the level of expression and density of the resistant subpopulation. This mechanism, generally known as indirect resistance, was first observed [61] with a strain of *Haemophilus influenzae* which was protected from the action of penicillin in the presence of β-lactamase-producing members of the *Enterobacteriaceae* family. Furthermore, indirect resistance to β-lactams has been widely linked to numerous antibiotic treatment failures where β-lactamase producers protected a susceptible pathogen [62–64].

These studies clearly show that antibiotic degraders have the potential to protect susceptible bacteria and avert the propagation of resistance. However, a closer look also reveals that dissemination of these genes can have harmful consequences for human and animal health.

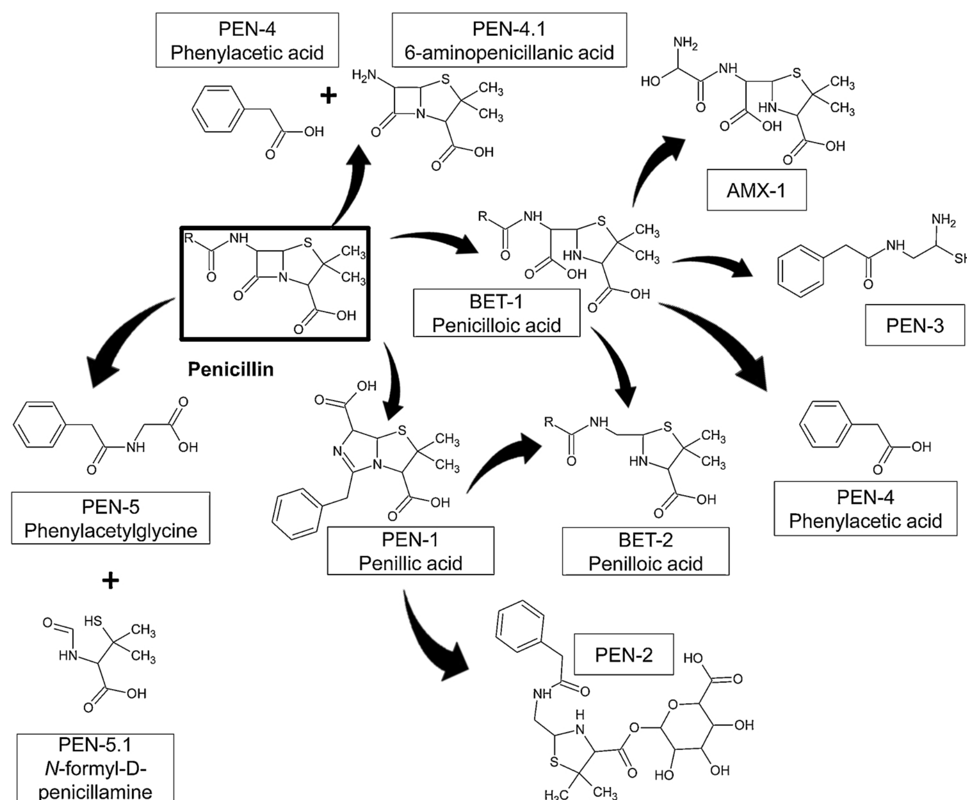

Fig. 1. Summary of main products of penicillin G (PEN-1 to 5.1) and amoxicillin (AMX-1). Metabolites with BET nomenclature have been found for both penicillins (G and amoxicillin).

Thus, the use of antibiotic degraders should be carefully evaluated before considering biotechnological applications. On the one hand, some authors claim that antibiotic degradation or transformation is an energetically expensive metabolic trait, with a high fitness burden for the host and, therefore, unlikely to disseminate and become fixed in a given population [65]. On the other hand, as many subsequent studies have shown, under continuous selective pressure, that even genes with high fitness cost are likely to persist in natural populations due to compensatory mutations, horizontal gene transfer and co-selection events [65]. The results of these studies call for diligence when considering the use of antibiotic degraders as bioremediation tools.

The impact of hydrolyzed β-lactams on receiving waters and soils and their possible influence on the development or spread of antibiotic resistance has also not been assessed. Although β-lactams are the most heavily used antibiotics in both human and veterinary medicine, little effort has been applied to the development of technologies able to simultaneously eliminate β-lactam residues and prevent the dissemination of resistance [66]. There is an urgent need to gain further knowledge in this field since research on new β-lactam sub-classes has been steadily declining and, currently, no other antibiotic class rivals their efficiency and safety [67].

Macrolides

Macrolides exert their antimicrobial action through binding to the nascent peptide exit tunnel close to the peptidyl transferase center of the large ribosomal subunit, preventing the exit of the growing peptide chain [68]. Due to their frequent detection in water bodies throughout Europe [69–73], erythromycin, clarithromycin, and azithromycin have been added to the watch list of emerging water pollutants by the European Commission [74] and are considered to be emerging pollutants worldwide [75,76] (see suppl. Table S2 for detailed physicochemical properties). Resistance to macrolides occurs mainly through enzymatic inactivation (hydrolysis and phosphorylation), and efflux pumps [77]. Detailed information on the different members of this class, their use and application spectrum can be found elsewhere [78,79].

Abiotic degradation

Abiotic degradation of erythromycin frequently occurs under strongly acidic conditions, which cause the loss of a water molecule and it is commonly reported in the literature as erythromycin-H_2O (ERY-d, suppl. Figure S3) [70,80]. Erythromycin-H_2O is often detected in environmental samples, and it can be found at higher concentrations than the original parent compound (μg/L), as demonstrated for surface water in the U.S. [92]. Although this dehydrated erythromycin has negligible antibiotic activity, it has been reported to select for resistant bacteria in sequencing batch reactors inoculated with activated sludge [81].

Erythromycin can also undergo oxidative transformation through a sulfate radical-based oxidation process under UV-irradiation, but the degree of degradation was dependent on the chemical composition of the water matrix [82]. Ozonolysis was also shown to degrade effectively macrolide antibiotics such as clarithromycin, azithromycin and roxithromycin into biologically inactive forms [83,84]. Solar radiation also induced photolysis of macrolide antibiotics, namely erythromycin and roxithromycin [83]. This in-depth study concluded that the extent of photolysis was higher in natural water samples than in reverse osmosis deionized water, probably due to the presence of dissolved organic carbon in the natural water that can act as photosensitizer [83]. For erythromycin, a photodegradation pathway was proposed (suppl. Figure S3). The effect of photosensitizers was demonstrated in an azithromycin photodegradation experiment [85], in which it was

demonstrated that nitrate or humic acid supplementation of fresh water enhanced photodegradation greatly (5 and 16 fold).

Biotransformation and biodegradation

A CBT was performed with effluent from a sewage treatment plant [38]. There, erythromycin was tested under OECD guidelines and found not to be readily biodegradable. Further tests with activated sludge (ZW) were conducted with higher inoculum concentrations and a lower ratio of substance to cells which provide more favorable conditions to assess the inherent biodegradability [36]. In this study, less CO_2 was produced in the presence of erythromycin than in controls without the antibiotic, indicating an inhibitory effect of the compound on biologic activity in the sludge. Another azithromycin biodegradation test according to OECD 308 [86] was performed with aerobic and anaerobic sediment and water samples from Maryland, USA [87]. In this test, azithromycin did not undergo significant biodegradation either in aerobic or anaerobic conditions.

All the aforementioned tests relied on previously unexposed microbial communities. Thus, the results did not always reflect the real potential for biotransformation of a given antibiotic by environmental microbiota. In fact, in one study [88], faster dissipation was reported of all macrolides tested in soil that was previously exposed to the antibiotics in contrast to the non-exposed soil. This result suggests that long-term acclimatization can play an important role. Furthermore, mineralization of erythromycin by the microbial community of aquaculture sediments has been observed [89] and subsequently a mineralization test was reported on pre-exposed sediments compared to sediment that had not been exposed to any antibiotics [90]. The study showed that erythromycin added to the sediments resulted in no changes in the number of aerobically grown bacteria in the microbial community; however, it did influence its composition by favoring the proliferation of bacteria harboring specific degradative enzymes, such as erythromycin-esterases, encoded by *ere*A and *ere*B. These enzymes catalyze the cleavage of the 14-membered lactone ring, as depicted in Fig. 2, and leads to the loss of antimicrobial activity (ERY-1) [91].

Macrolides can also be modified through enzymatic phosphorylation by macrolide 2′-phosphotransferase (E.C. 2.7.1.136) [92,93]. This inducible and intracellular enzyme catalyzes the hydrolysis of the lactone ring (ERY-2, Fig. 2) and shows the highest activity against various 14-membered ring macrolides (e.g., erythromycin) and the lowest against its 16-membered-lactone ring counterparts (e.g., tylosin, suppl. Table S2).

Few studies have assessed the impact of these antibiotic degraders in natural populations. To date, only one group has reported indirect resistance of susceptible populations in the presence of *ere*A expressing bacteria [60]. Despite being fairly rare among clinical isolates [77], these genes have been detected in mobile genetic elements [94] indicating a high risk of horizontal gene transfer. Therefore, as for other classes of antibiotics, bioremediation techniques making use of these strains should be avoided due to their potential to further aggravate the burden of resistance. Nevertheless, since both EreA and EreB are hydrolytic and do not require complex co-factors, treatment with enzyme preparations could provide a solution to treat contaminated sites and to prevent the development and spread of resistance.

Quinolones

Quinolones act on two enzymes essential for DNA replication and transcription, topoisomerase II (DNA gyrase) and topoisomerase IV [2]. Currently, first- generation quinolones (nalidixic acid, flumequine and oxolinic acid, suppl. Figure S4) are scarcely used, whereas fluoroquinolones (second generation onward) are still highly relevant to human therapy but less frequently used in veterinary medicine [6,95,96]. These molecules are found in the environment as they are mostly excreted in unchanged form and released into wastewaters and soil through human excretion and manure dispersion [97,98]. Fluoroquinolones, mainly ciprofloxacin, have frequently been detected in

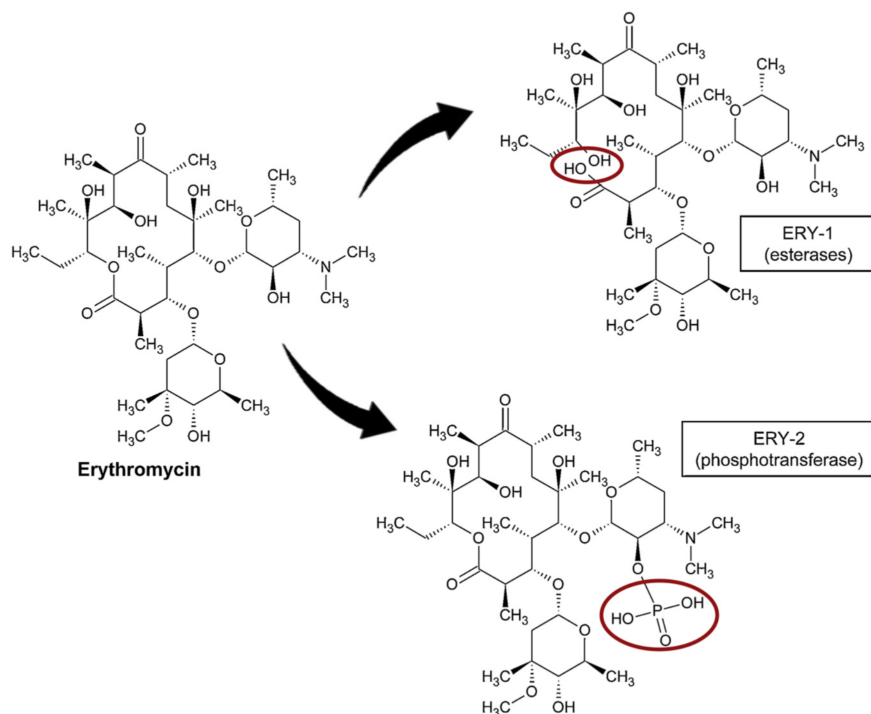

Fig. 2. Enzymatic modification of erythromycin, a 14-membered ring macrolide. Red circles mark the cleavage of the ring (ERY-1) by esterases and transformation by phosphotransferases (ERY-2).

hospital wastewaters, urban wastewater treatment plants (WWTPs) and also in surface waters, at μg/L and ng/L quantities respectively [99]. However, the sorption of fluoroquinolones to organic matter is very high and they are often detected at higher concentration in soil in comparison to the aquatic environment [99–102]. For instance, in WWTPs both ciprofloxacin and norfloxacin were reported to be eliminated by sorption to activated sludge (approx. 50%) and consequently accumulate in sludge-treated soil over long time periods [100]. 21 months after sludge application residual amounts of these antibiotics (μg/kg levels) could still be detected in soil, demonstrating their reduced mobility and recalcitrance to biological degradation.

Resistance occurs mainly through target modification (DNA gyrase A), efflux and, more recently described, acetylation of the piperazine ring by an aminoglycoside acetyltransferase (AAC(60)-Ib-cr) [103]. More detailed information on quinolones can be found in [2,104] (suppl. Figure S4 and Table S2).

Abiotic degradation

Fluoroquinolones are extensively transformed by photolysis and by photo-catalytic processes [19,105]. In water matrices under natural sunlight they can undergo many different reactions including (suppl. Figure S5) oxidative defluorination (CIP-a), oxidation of the piperazine ring (CIP-b) and consecutive degradation of the oxidized ring (CIP-b.1 and b.2), reductive defluorination (CIP-c) and degradation of the piperazine ring (CIP-d) [105]. These photo-transformation reactions were also shown to occur in soil [106] and can potentially be mediated by naturally occurring catalysts (e.g., manganese-oxide) [107]. Although the antimicrobial activity of these intermediates decreased due to defluorination, it was not completely eliminated, since the quinoline core structure remained intact [108]. Thus far, quinoline ring degradation and complete loss of antimicrobial activity have only been reported for advanced (photo)oxidative processes mediated by TiO_2 or by different iron species [109–111].

Biotransformation and biodegradation

Fluoroquinolone antibiotics are easily eliminated by adsorption to sediments and transformed by photolysis. However, they appear to be recalcitrant to degradation in tests with non-acclimatized microbial communities (activated sludge or soil) as shown in Closed Bottle tests [37,38], Zahn Wellens tests, CO_2-evolution tests [36] and with ^{14}C-labelled fluoroquinolones in soil [112]. However, considerable information is available on different pathways for transformation and degradation of these drugs by single microorganisms (suppl. Table S3). These pathways were recently reviewed [113–115] and since then, only a few more studies have been published on this topic. This section provides an overview of the general mechanisms, updating current knowledge and investigating the biotechnological potential of these microbial strains.

Quinolones of the second generation are the most frequently studied and were shown to undergo a number of different modifications, see suppl. Table S3 for a summary of all organisms and metabolites; Fig. 3 for main reactions found for ciprofloxacin and other fluoroquinolones; and suppl. Figure S6, for transformations specific to enrofloxacin, norfloxacin, ofloxacin and danofloxacin. These include oxidative defluorination (FQ-4), demethylation (FQ-8), breakdown and loss of the piperazine ring (FQ-1.1, FQ-2, FQ-6, FQ-7.1 and 7.2, CIP-4 to 7), hydroxylation of the quinoline nucleus (FQ-4.1 and 5), oxidative decarboxylation (FQ-3), *N*-oxidation (DAN-1 and ENR-2), *N*-acetylation (FQ-1) and loss of ethylene in the piperazine ring (C_2H_4, FQ-6). First (nalidixic acid and flumequine) and fourth-generation quinolones (moxifloxacin and pradofloxacin) are transformed by mechanisms similar to those of the second generation of this class. However, as shown in Fig. 4, some additional reactions are unique to first-generation quinolones due to the lack of the piperazine ring. For instance, nalidixic acid can undergo hydroxylation and oxidation in the methyl group attached to the C7 carbon of the quinoline nucleus (NAL-1 and 2, Fig. 4), whereas this reaction cannot occur in other fluoroquinolones due to the presence of the piperazine ring in this position. Furthermore, flumequine, with a fused ring between the nitrogen and the C8 carbon, undergoes hydroxylation (FLU-1) and oxidation (FLU-1.1) on this fused benzene ring (Fig. 4). Curiously, the transformation of fourth-generation quinolones has only been reported for versatile degraders, such as the brown-rot fungus *Gloeophyllum striatum* [113] (Fig. 5), and the bacterial strain *Labrys portucalensis* F11 [116], which can partially defluorinate moxifloxacin and other second-generation quinolones.

Of all the mechanisms reported to date, *N*-acetylation is by far the most common route for fluoroquinolone transformation. This reaction can be catalyzed by numerous bacterial and fungal strains [117–123], and is also a typical route for detoxification in humans and animals [124,125]. Interestingly, a variant of aminoglycoside acetyltransferase (AAC(6′)-lb), an enzyme initially linked to aminoglycoside resistance, was recently also shown to *N*-acetylate ciprofloxacin, making it the first enzyme able to modify antibiotics of two different classes [126].

Including *N*-acetylation, most of the transformations described so far result in a marked decrease in antimicrobial activity [113], but information on the enzymes and genes involved in these processes is scarce. For instance, the degradation of fluoroquinolones by *G. striatum* may be mediated by hydroxyl radicals, since the intermediates were similar to those detected during catalysis [127,128]. The production of hydroxyl radicals and subsequent oxidation of xenobiotics is a commonly reported ability of ligninolytic enzymes [129,130]. However, no evidence was gathered so far that these enzymes could be involved in fluoroquinolone oxidation by basidiomycetes (class *Agaricomycetes*) [121,131]. Furthermore, in one study [144], the presence of the antibiotic increased the expression of enzymes involved in cellulose and hemicellulose hydrolysis, suggesting that these types of enzymes may play an important role in the breakdown of fluoroquinolones by the basidiomycete *Pleurotus ostreatus*. Although ligninolytic enzymes may not be expressed by basidiomycetes during quinolone degradation, studies with the purified laccase have shown that it can partially eliminate these molecules [132,133]. This process can reduce their antimicrobial activity, although no metabolic pathway has been proposed [132].

Despite the complex network of reactions involved in fluoroquinolone transformation, mineralization of these molecules is limited since the quinoline nucleus is rarely cleaved (see Figs. 3,4,5). High throughput studies suggested an abundance of quinolone-subsisting microorganisms [48,49]. However, only two microbial strains reported to date can mineralize these drugs significantly: *Curvularia lunata* [123] which converted at least 30% of the initial ^{14}C-danofloxacin into CO_2 and *G. striatum* which mineralized approximately 20% of ^{14}C-enrofloxacin or ^{14}C-ciprofloxacin [127,128]. For the first strain the remaining radioactivity was recovered from culture extracts suggesting an accumulation of intracellular metabolites or incorporation of the parent drug onto the biomass, but since no metabolites were identified, a metabolic pathway could not be proposed. Nevertheless, in both cases, the ^{14}C label was within the quinoline nucleus, providing proof of partial degradation of the core structure.

To our knowledge, very few studies have addressed the removal of these contaminants using bioaugmentation or bioremediation approaches. For instance, removal of 0.5 mg/L ciprofloxacin from synthetic wastewater was enhanced in a 3-stage membrane bioreactor (MBR) by combining it with a bioaugmentation process [134]. The system consisted of an entrapped cell tank (first stage) with polyvinyl alcohol and sodium alginate beads of *Pseudomonas putida* strain TISTR1522, a known aromatic compound degrader, an anoxic tank without stirring (second stage) and an aerobic MBR tank (3rd stage). This strategy significantly enhanced ciprofloxacin removal up to 90%, and at least 24 metabolites could be detected. These intermediates were very similar to those proposed by others mainly in photolysis studies,

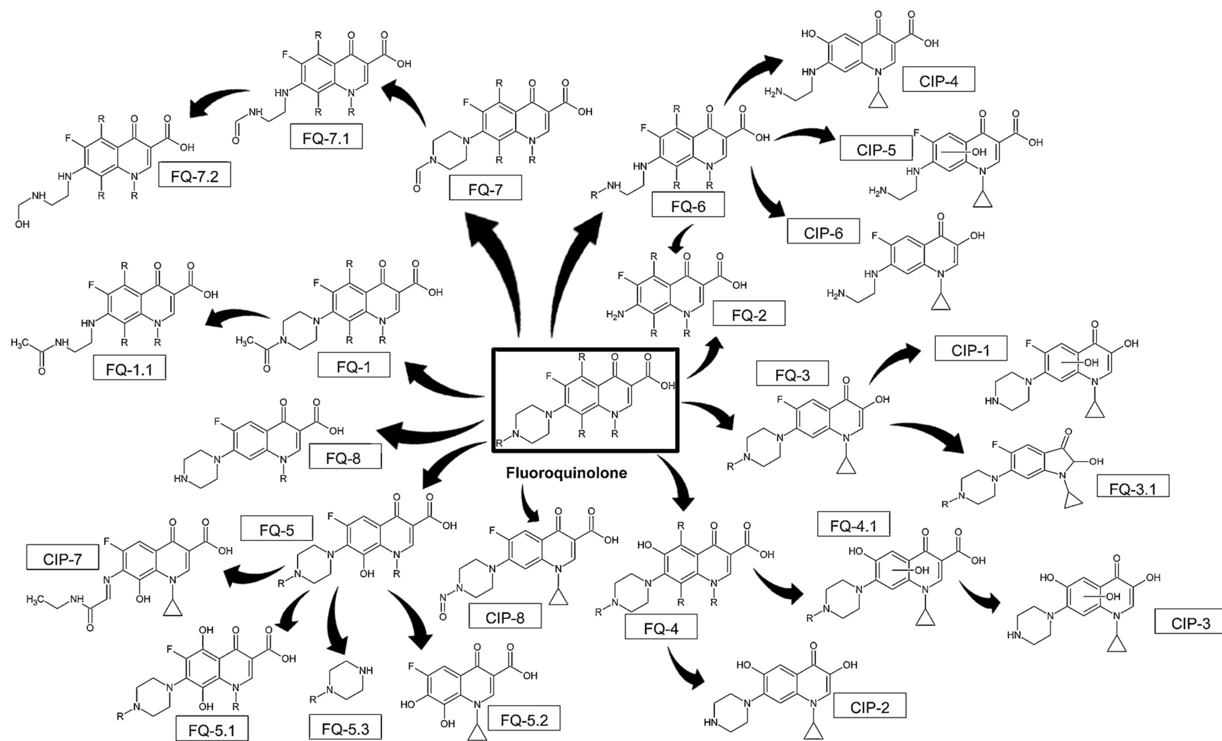

Fig. 3. Main pathways for the transformation and degradation of 2nd generation quinolones by bacterial and fungal species. FQ metabolites are found for two or more fluoroquinolones and CIP metabolites are found only for ciprofloxacin.

and they resulted from: (i) oxidative defluorination, (ii) breakdown and cleavage of the piperazine ring, (iii) decarboxylation and (iv) possible cleavage of the quinoline core. This study lacked a detailed structural characterization of these metabolites, but it highlights the benefits of a bioremediation strategy to enhance the removal of these drugs in real settings.

In summary, biological degradation of quinolones can be accomplished by many bacterial and fungal strains. However, there is a lack of

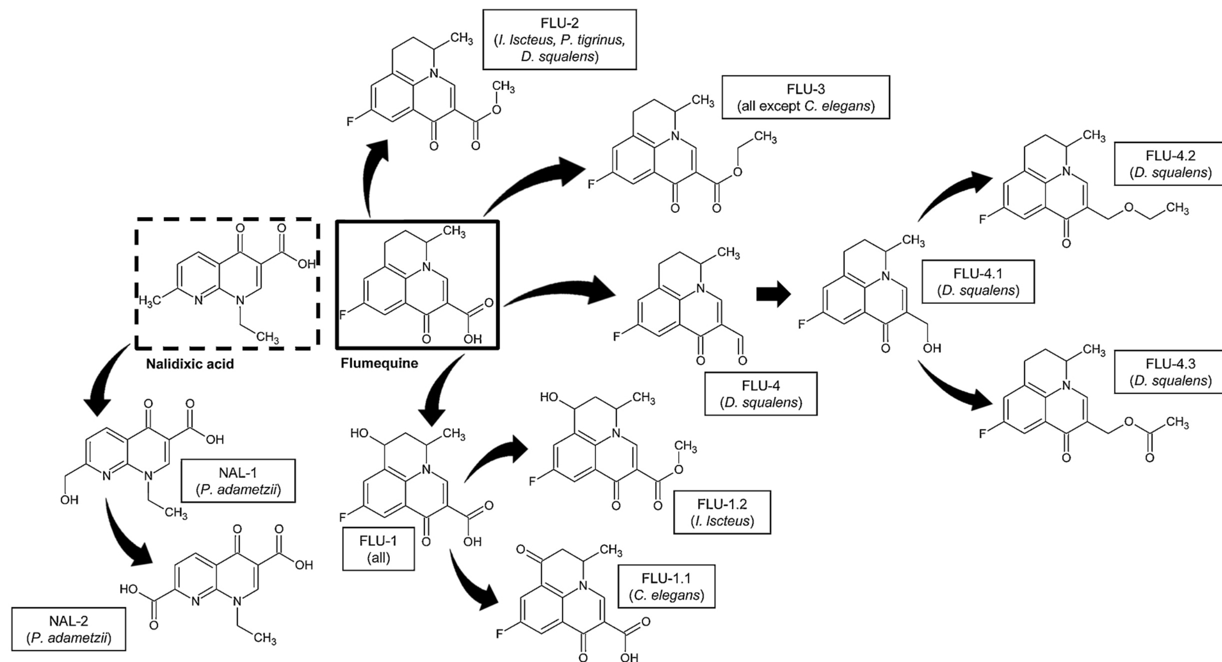

Fig. 4. Main pathways for flumequine and nalidixic acid degradation by different fungal species (1st-generation).

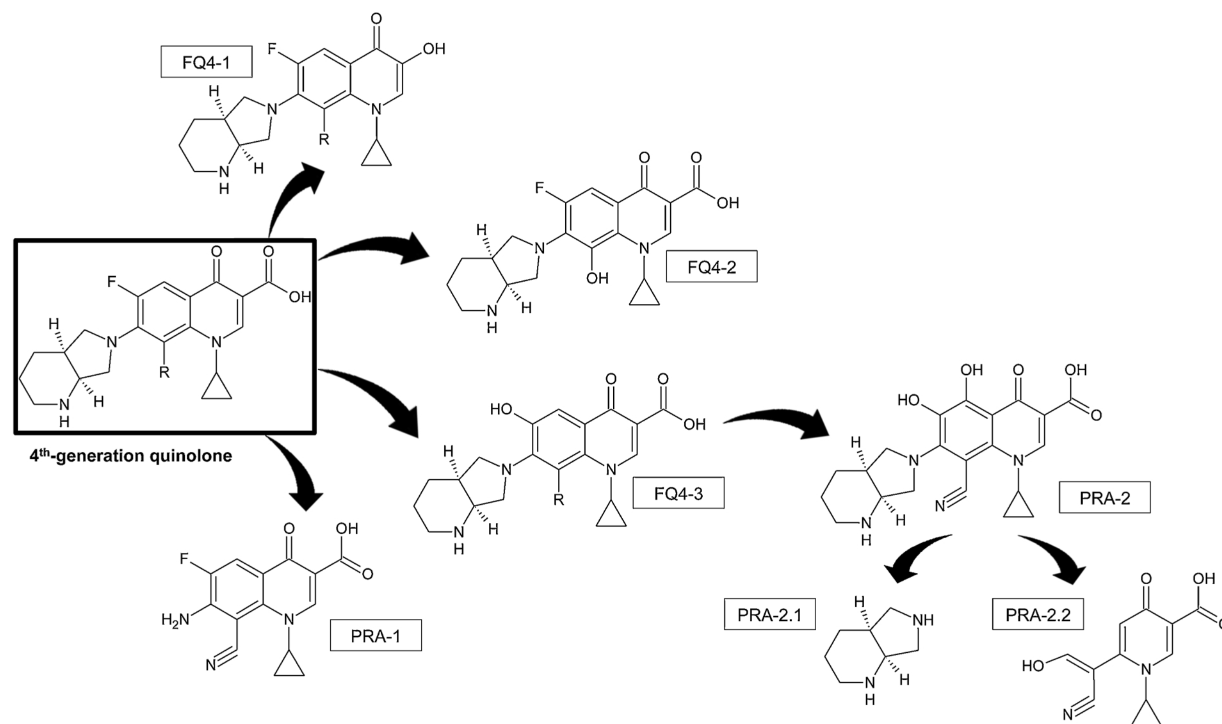

Fig. 5. Main pathways for degradation of moxifloxacin and pradofloxacin, 4th-generation quinolones, by *G. striatum*.

studies focused on the molecular basis of the degradation mechanisms and application of the degraders in real settings, and the enzymes involved in these processes are still unknown. The link between degradation and resistance has also not been elucidated for these antimicrobial agents.

Ionophore antibiotics

Ionophore antibiotics form complexes with essential cations (K^+, Na^+, Ca^{2+}, Mg^{2+}) and can easily cross membranes of susceptible bacteria through cation/proton (H^+) exchange [135–137]. Due to their mechanism of transport, ionophores can disrupt ion gradients in the cell and lead to the exhaustion of cellular energy and cell death [138–140]. They are not applied in human therapy [141], but in 2015 in the USA, more than 4740 t were applied as growth promoters in animal husbandry [142]. To date, resistance appears to be intrinsic and mediated by extracellular polysaccharides (glycocalyx) [137]. Moreover, no specific genes have been linked to an increased tolerance [137]. Because of this lack of evidence regarding the molecular mechanism of resistance, there is an on-going debate as to whether resistance against these agents can be acquired through horizontal gene transference [137,140,143]. Furthermore, the presence of heavy metals, which has been frequently linked to the development and spread of antibiotic resistance, has not been studied in the context of ionophores [144–146]. There are few studies on the occurrence of members of the ionophore class in the environment, but they were shown to be readily detectable in the compartments tested [147,148]. More detailed information on ionophore antibiotics can be found in [149].

Abiotic degradation

Due to their structural diversity, stabilities of the different representatives vary (see suppl. Table S2 for main physicochemical properties). While lasalocid was found to be rather stable for five days in aqueous conditions at pH values 4, 7 and 9, monensin, due to hemiketal (C9) and ketal (C25) centers, was susceptible to hydrolysis at pH 4. Salinomycin and narasin, presumably also due to ketal centers at C17 and C21, also hydrolyzed both at pH 4 and 7 while being stable at pH 8 [150]. Another study proposed structures of the products of monensin and salinomycin hydrolysis. For monensin, hydrolysis gave rise to a diastereomer that could be re-transformed to the parent compound (MON-a), to a ring cleavage product opened at the hemiketal centre (MON-b) and a product resulting from loss of water (MON-c, suppl. Figure S7). Salinomycin hydrolysis resulted in a ring opening at the ketal center at C21 (SAL-a), two products of fragmentation and significant rearrangements within the molecule (SAL-b and SAL-c, suppl. Figure S7). It was claimed that antimicrobial properties decreased as hydrolysis progressed but that some intermediates of the reaction would retain antimicrobial activity [151]. Based on spectrophotometric studies it was concluded that of the four mentioned ionophores, only lasalocid was susceptible to photolysis, as an aromatic ring (C2 through 7) in the structure caused significant absorption around 300 nm [150]. In contrast, treatment with UV/H_2O_2 led to rapid degradation of monensin, salinomycin, and narasin (the half-lives of all three compounds were around two minutes), indicating that reactions with hydroxyl radicals formed from H_2O_2 upon UV irradiation [151].

Biotransformation and biodegradation

Studies on the biodegradation of ionophores have led to ambiguous conclusions. Closed-bottle tests (Zahn-Wellens test) did not show biodegradation of monensin in activated sludge [36], while degradation of monensin in soil could be positively correlated to the content of organic matter as well as to the water content of the soil [152]. It was found that the half-life of ionophores in unspiked samples was less than a week in a clay loam soil, but three weeks in loam soil samples. Another study with unspiked soils showed that degradation of salinomycin and monensin in non-fertilized soil microcosms was mainly due to abiotic

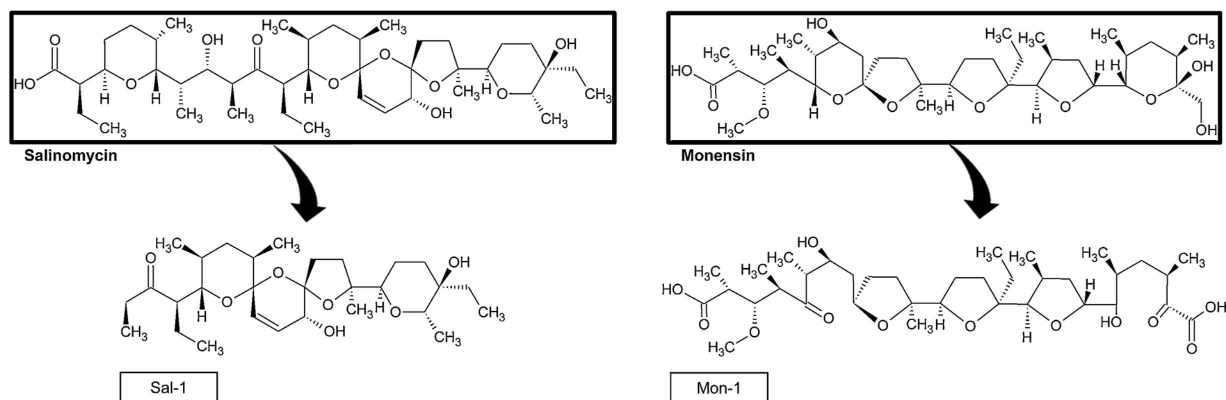

Fig. 6. Biodegradation products of salinomycin and monensin.

reactions. Biodegradation only contributed to the removal of these compounds when soil microcosms were fertilized with poultry litter. Biodegradation of monensin in soil resulted in the oxidative cleavage of the ether rings containing C3 and C29 [153]. Other studies on the degradation of ionophores during composting indicated that the extent depended on both the respective compound and the applied matrix [141,154]. Neither the composting of poultry litter nor of dairy manure led to an increase in removal rates of monensin contained in each. In contrast, composting of poultry litter and dairy manure accelerated removal of lasalocid,both by > 90% in 12 weeks, respectively, and composting dairy manure decreased salinomycin concentrations by > 90% in 4–6 weeks [141]. It was shown [154] that both lasalocid and monensin were degraded by around 90% within 10 days in soil samples spiked with 2 mg/kg, and that their removal could be almost exclusively attributed to biological activity. Two bacterial strains capable of abolishing antibiotic activity of salinomycin isolated from soil samples were *Pseudomonas stutzeri* FH1796 and *Enterobacter agglomerans* FH1793 [155]. The former was capable of degrading salinomycin through a retroaldol cleavage leading to the loss of the corresponding ring (Fig. 6, Sal-1). The reaction product of this step was later also found in soil degradation tests [153]. In the same study, incubation of monensin and broiler litter yielded a biotransformation product resulting from hydrolysis at C25 and hydroxylation at C26 (Fig. 6, Mon-1).

Others

For other antibiotics, there is little information on stability, degradation, and biodegradation. Due to the relatively low amounts prescribed compared to the more common classes, their respective environmental impacts may not be regarded to be as important. Nevertheless, they deserve attention as many represent antibiotics of last resort. Here, single members of antibiotic classes not mentioned above are discussed as a group.

Oxazolidinones

Generally, oxazolidinones (see suppl. Table S2, for main physicochemical properties) inhibit bacterial protein synthesis by preventing the formation of N-formyl-methionyl-tRNA–ribosome–mRNA ternary complex [156]. Background information on oxalidinones, a group containing last-resort antibiotics, i.e. history, spectrum of application, and resistance mechanism can be found in [157]. Resistance has occasionally been reported, [158].

Abiotic degradation

Linezolid was shown to be stable in aqueous solutions, but could be partially degraded by HCl, NaOH, H_2O_2, heat (80 °C) and UV irradiation (365 nm) [159]. Likewise, exposure to HCl, NaOH, H_2O_2 and UV irradiation led to the degradation of tedizolid phosphate [160]. In simulated gastric fluid (0.1 M HCl) at 37 °C, it had a half-life of 63 days [161].

Biotransformation and biodegradation

No data on biotransformation and biodegradation is available.

Nitroimidazoles

Members of this class are taken up by bacteria before conversion into a nitroso free radical. The activated molecules bind nonspecifically to bacterial DNA, causing DNA damage and inhibition of synthesis [162]. The class contains several compounds sharing similar structures based on a 5-nitroimidazole ring, including metronidazole, dimetridazole, and tinidazole (see suppl. Table S2). Metronidazole is a model for this class, which is widely used due to its effectiveness against both anaerobic bacteria and even protozoa [162]. Also here, resistance is frequently observed [163].

Abiotic degradation

Both dimetridazole and metronidazole were shown to be photocatalytically degradable by UV-irradiation of TiO_2, yet no degradation products were described in either case [164–166].

Biotransformation and biodegradation

Metronidazole was shown to be biodegradable in aerobic soil-manure slurries [167] with a half-life of 13.1–26.9 days. However, the authors claimed that these biodegradation rates might be overestimations of processes taking place in the environment. No transformation products have been reported yet.

Glycopeptides

Glycopeptides inhibit peptidoglycan synthesis, by binding to acyl-D-Ala-D-Ala groups in cell wall intermediates [168]. Their biochemistry has been described in detail in [168], while mechanisms of resistance have also been described [169]. Within this class, vancomycin (see Table S2, for main physicochemical properties) is widely used to treat

severe infections caused by Gram-positive bacteria, such as *Clostridium difficile* [169,170] and methicillin-resistant *Staphylococcus aureus* (MRSA). Despite its use as a drug of last resort, reports of resistance have been accumulating [169].

Abiotic degradation

Vancomycin was shown to hydrolyze even at neutral pH, leading to more than 10% loss within 5 days at pH 7.1 [171]. No transformation products have been reported.

Biotransformation and biodegradation

A study reported that vancomycin was not biodegradable in the Zahn-Wellens test using activated sludge as inoculum [36].

Lincosamides

Lincosamides (see suppl. Table S2) act on the 50S subunit of bacterial ribosomes, inhibiting protein synthesis [172]. Biochemistry of and resistance mechanisms against lincosamides have been described in [172,173].

Abiotic degradation

Clindamycin was shown to be degradable by a combination of zero-valent iron, H_2O_2 and ultrasonication [174], while being stable in aqueous solutions, even at extreme pH values [175].

Biotransformation and biodegradation

There is little information on the biotransformation of lincosamides. A resistance gene was found in *Enterococcus faecium* HM1025 to inactivate clindamycin by adenylation (Fig. 7, Cli-1) [176]. Moreover, incubation of clindamycin in soil showed the transformation of clindamycin to clindamycinsulfoxide Cli-2), *S*-desmethyl clindamycin (Cli-3), hydroxyl clindamycin sulfoxide (Cli-4) and *N*-desmethyl clindamycin (Cli-5), respectively (Fig. 7) [177], which appeared to be persistent. As no sterile controls were analyzed, these transformations can only be tentatively attributed to biotransformation. Moreover, the transformation products were not tested for antimicrobial activity, so the effect on the transformations of these compounds on the propagation of resistance cannot be effectively evaluated.

Lipopeptides

Lipopeptides generally act by integrating into membranes and disrupting membrane potential. They form various subgroups, of which daptomycin and polymyxin B are approved by the US FDA. Resistance occurs mainly through target modification (membrane lipopolysaccharides and lipid A) [178]. Background information on them has been compiled in [179].

Abiotic degradation

Daptomycin is prone to ester hydrolysis under alkaline conditions (Fig. 8, cleavage site a), aspartyl transpeptidation in the pH range 3–6 (Fig. 8, Dap-1 and Dap-2) and degradation by unknown mechanisms at low pH values [180].

Biotransformation and biodegradation

Further reports showed that daptomycin could be cleaved by hydrolysis or deacetylated enzymatically by certain resistant actinomycete isolates, rendering them inactive (Fig. 8, hydrolysis cleavage sites a–d) [181]. Other studies also confirmed enzymatic hydrolysis of surfactin (another lipopeptide) to be catalyzed by *Streptomyces* sp. Mg1 [182].

Quinoxaline-*N,N′*-dioxides

Olaquindox shows antimicrobial activity and is used as a medicinal feed additive in pig, chicken and calf husbandry [183]. It is believed to act by covalent binding to DNA and subsequent inhibition of DNA

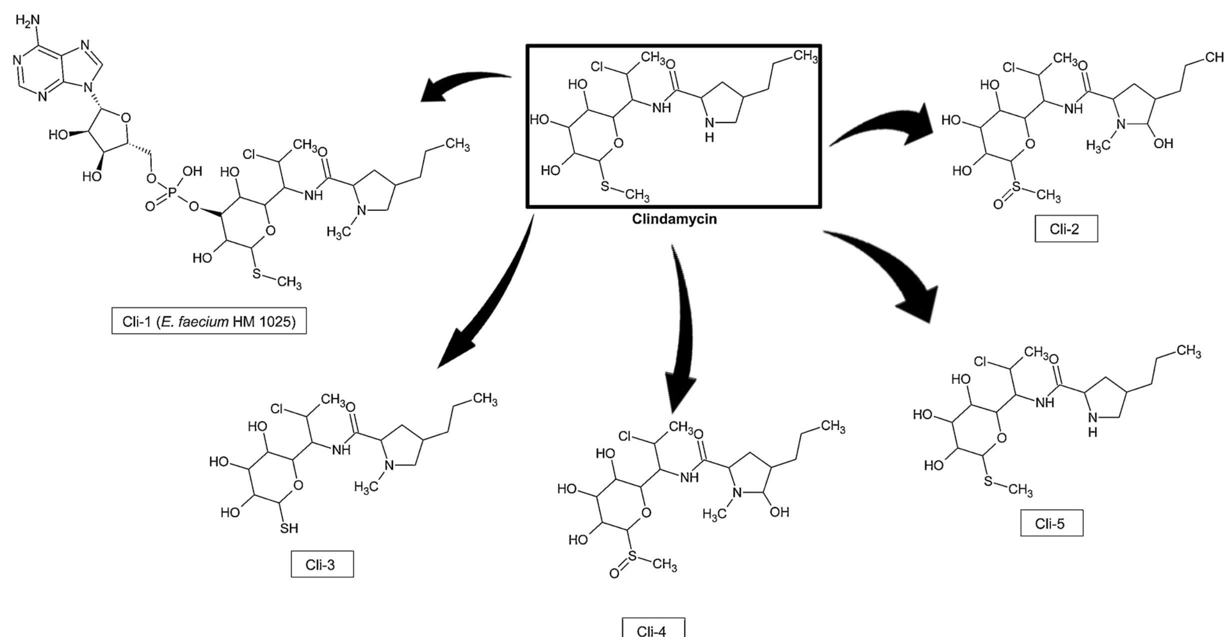

Fig. 7. Clindamycin and biotransformation products.

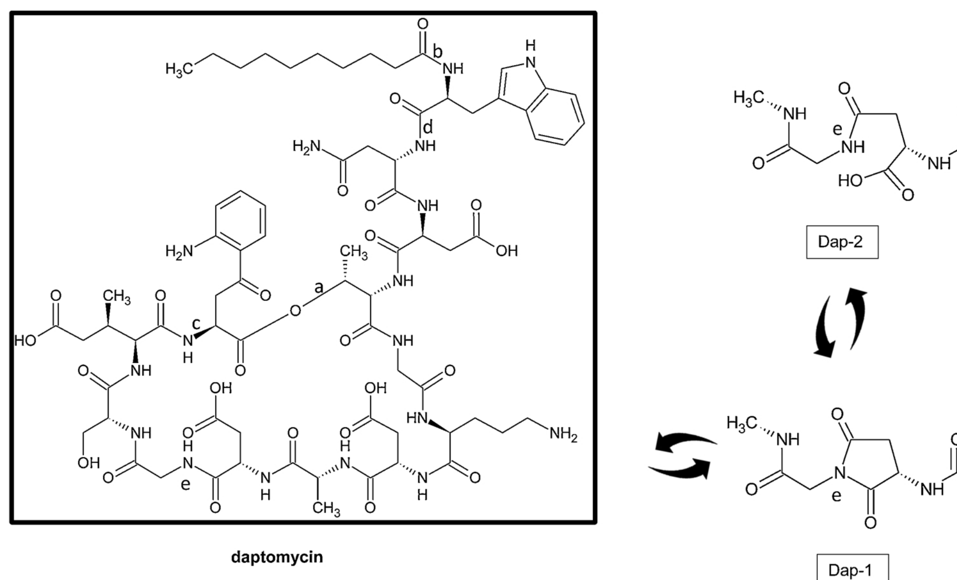

Fig. 8. chemical structure of daptomycin (letters a through d indicate preferential cleavage sites of biological degradation, e indicates site for aspartyl transpeptidation resulting in Dap-1 and Dap-2).

synthesis [184]. Selective pressure exerted by its use among livestock was shown to contribute to the selection and dissemination of multiresistant features among bacteria [185,186]. Resistance can occur by active transport through multidrug efflux pumps [187].

Abiotic degradation

Olaquindox can be hydrolyzed in aqueous solutions at 80 °C, especially under acidic conditions. It was also unstable under oxidative stress (30% H_2O_2) and UV irradiation, where it formed 3-methyl-2-hydroxylquinoxyline-4-oxide as the main degradation product (Ola-1, suppl. Figure S9,) [188]. It was further found to sorb only weakly to different kinds of soils, with slightly higher sorption coefficients for sandy loam than for sandy soils [189]. Further studies in soil interstitial water under anaerobic conditions showed that only 8% of the initial concentration remained after 21 days, while the potency of the samples decreased accordingly [190]. The authors assumed that the transformation was due to the compound's photosensitivity.

Biotransformation and biodegradation

Aerobic incubation of olaquindox in soil-manure slurries led to swift transformation rates, with a half-life of 5.8–8.8 days [167]. The degradation was neither significantly influenced by the type of soil (sandy or loamy), nor by the content of manure (0, 1 or 10% V/W) [167].

Streptogramins

Streptogramins consist of pairs of molecules (see suppl. Table S2, for properties of pristinamycin and virginiamycin molecule pairs), each of which block gene translation by binding to the bacterial 50S ribosomal subunit. Resistance can occur by ribosomal target modification, efflux and enzymatic modification [191]. Background information on this class of last-resort antibiotics has been described in [191,192].

Abiotic degradation

Limited information on the stability of streptogramins is available. One study documented the instability of pristinamycin in 0.5 M HCl, 0.5 M NaOH and 3% H_2O_2, respectively [193]. Degradation was also observed after exposure to UV light (254 nm). Another study investigated the stability of virginiamycin in alcohol fermentation batches, but found no significant degradation during 72 h between pH 3.8 and 4.8, either at 25 °Cor 35 °C [194].

Biotransformation and biodegradation

Virginiamycin was shown to be moderately to rapidly degradable in different tested soils. Within 64 days, extensive primary degradation had been observed, in addition to 12–40% of mineralization [195]. Culture supernatants of laccase-induced *Aureobasidium pullulans* strains were found to inactivate virginiamycin (6 mg/l) within 24 h [196]. Type A streptogramins can also be inactivated by *O*-acetylation by several acetyltransferases and type B streptogramins were reported to be linearized and inactivated by lyases (suppl. Figure S10) [191].

Conclusions

Here antibiotics of the major and minor classes have been examined. Most were found to be susceptible to both biodegradation and biotransformation reactions, but, as observed previously [1], the subsistence phenotype is quite rare and poorly documented:

- **β-lactams:** mainly degraded by hydrolysis of the β-lactam ring, rendering degradation products that can often be detected at higher concentrations than the original form. To date, only few strains have been reported to further degrade the hydrolyzed product, and recently some enzymes and their encoding genes could be linked to this process. Beside β-lactamases, enzymes from different classes can also degrade β-lactams but their contribution to degradation in environmental samples has never been investigated.
- **Macrolides:** mineralization to a certain extent has been reported in soil. The underlying mechanism is probably linked to the action of esterases which were shown to partially hydrolyze the stable 14-membered lactone ring.
- **Quinolones:** there is extensive knowledge on the degradation of several antibiotics of this class. Degradation and transformation occur mainly in the piperazine moiety, while some fungal species

were shown to cleave the stable quinoline nucleus and even to partially mineralize some of these antibiotics.

- **Ionophore antibiotics:** some are reported to be degraded partially in soils or by isolated bacteria. However, the major concern is the lack of knowledge of whether resistance exists and whether cross-resistance is possible between this class of exclusive veterinary use and other classes that are primarily used for human medicine.
- **Others:** some groups were found to be susceptible to bio-transformation and biodegradation, but the metabolic pathway and microorganisms are rarely described. Most common reactions include adenylation (lincosamides), deacetylation, hydrolysis (lipopeptides), acetylation and linearization (streptogramins).

It has become evident that future studies on the removal of antibiotics in any environmental compartment should extend beyond the point where the mere presence or absence of the parent compound is investigated. A great deal has been learned about the reversibility of many transformation reactions and the antibiotic activity or other forms of toxicity emanating especially from various transformation products. With that in mind, solely focusing on the parent compound is shortsighted, as several agents with a potentially great impact on the propagation of established resistance mechanisms and the formation of novel ones remain unnoticed. Major transformation products should be included in studies whether they possess antibiotic potential themselves or can be re-transformed to their respective parent compounds. In this way, a meaningful assessment can be better performed of the environmental fate and impact of these compounds and potential risks that may have remained hidden so far.

Generally, there is also still a lack of knowledge on the genetic determinants of the documented degradation mechanisms. In fact, except for β-lactamases, which are widespread and highly prevalent, only a few genes have been well described to date.

Investigation of resistance mechanisms has often overlooked the impact of biodegradation in the dissemination of these resistance determinants. This has hindered understanding of the interplay between antibiotic resistance and degradation, which is of special concern since in recent years antibiotic degraders were found to have profound impact on the development of resistance. Among them, the best-known phenomenon is that of indirect resistance, which may lead to unexpected antibiotic therapy failures as non-pathogenic degrading bacteria could protect susceptible pathogenic microorganisms [60]. Thus, future studies should assess both the effect of biodegradation on the development of new resistance mechanisms and their effect on the propagation of known resistance determinants. This new knowledge could help understanding of whether the design of biodegradable antibiotics can help delay the onset and spread of antibiotic resistance [197,198].

Acknowledgments

Ana Reis wishes to acknowledge the Portuguese Foundation for Science and Technology (FCT) for her PhD scholarship (grant reference: SFRH/BD/95814/2013). This work was financially supported by the Swiss National Science Foundation (Grant No. 160332), European Regional Development Fund (ERDF) and the Portuguese Foundation for Science and Technology (FCT) through projects POCI-01-0145-FEDER-006939 (Laboratory for Process Engineering, Environment, Biotechnology and Energy – UID/EQU/00511/2013) and NORTE-01-0145-FEDER-000005 – LEPABE-2-ECO-INNOVATION.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.nbt.2019.08.003.

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