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Susceptibility of archaea to antimicrobial agents: applications to clinical microbiology

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Abstract

We herein review the state of knowledge regarding the *in vitro* and *in vivo* susceptibility of archaea to antimicrobial agents, including some new molecules. Indeed, some archaea colonizing the human microbiota have been implicated in diseases such as periodontopathy. Archaea are characterized by their broad-spectrum resistance to antimicrobial agents. In particular, their cell wall lacks peptidoglycan, making them resistant to antimicrobial agents interfering with peptidoglycan biosynthesis. Archaea are, however, susceptible to the protein synthesis inhibitor fusidic acid and imidazole derivatives. Also, squalamine, an antimicrobial agent acting on the cell wall, proved effective against human methanogenic archaea. *In vitro* susceptibility data could be used to design protocols for the decontamination of complex microbiota and the selective isolation of archaea in anaerobic culture.

Keywords: Antimicrobial agent, archaea, methanogenic archaea, microbiota, susceptibility testing
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Introduction

Archaea form a distinct kingdom of life, in addition to eukaryotes, bacteria, and large DNA viruses [1,2]. Archaea comprise three phylogenetically distinct groups: the *Crenarchaeota* mainly consist of hyperthermophilic sulphur-dependent organisms, the *Euryarchaeota* contain methanogens and extreme halophiles, and molecular evidence has indicated the presence of the *Korarchaeota* in hyperthermophilic environments similar to those inhabited by the *Crenarchaeota* [3]. On the basis of their physiology, archaea can be organized into methanogens, extreme halophiles, and (hyper)thermophiles [4]. In addition to unifying features that distinguish archaea from bacteria, archaea exhibit other unique structural or biochemical characteristics related to their particular habitats [4]. Antimicrobial susceptibility patterns clearly distinguish archaea from the other organisms, and antimicrobials active against most bacteria are ineffective against archaea [5].

Methanogenic archaea (herein referred to as methanogens) are the sole organisms producing methane from $H_2 + CO_2$ [6]. They are widely distributed in nature in terms of their adaptation to different conditions of temperature, pH, and salinity, but remain confined to strictly anaerobic environments. The observation that human breathing released methane led to the isolation of the first human intestinal methanogen, *Methanobrevibacter smithii* [7]. Two other species, *Methanosphaera stadtmanae* [8] and *Methanomassiliicocus luminyensis* [9], were then isolated from the human gut microbiota by the use of anaerobic culture [10,11]. *Methanobrevibacter oralis* [12] was detected and isolated from periodontitis specimens, with the same procedure. Methanogens are perfectly adapted to their environment, and play a role in the oral and intestinal microbiota [13]. *M. smithii* is the dominant methanogen in the human gut, being detected with a high prevalence of 95.7%, whereas *Methanosphaera stadtmanae* and *Methanomassiliicocus luminyensis* are detected in 29.4% and 4% of individuals, respectively [14].

The gut is usually sterile at birth [15]. The development and establishment of the intestinal microflora is a complex process. It takes weeks or months to stabilize as a climax microflora, a process that is influenced by diet. Methanogens are detected until after weaning. It was generally reported that this marked change in diet was concomitant with an increase in the density and complexity of the microflora sufficient to produce the conditions that would allow further colonization by methanogens [15]. Studies of the genetic diversity of the human intestinal microbial community in relation to obesity, using culture-independent, molecular, phylogenetic and ecological statistical methods, showed that obese individuals have distinctly different intestinal communities than normal-weight individuals, confirming an association between methanogens and obesity [16,17]. Methanogens are also detected in the human vagina [18]. It was shown that diseased patients had a greater likelihood of being methanogen-positive, but no relationship was demonstrated between patient condition and the presence of methanogens in the vagina [18]. The potential role of archaea in digestive tract disease, obesity and vaginal infection has not been firmly established [17–19], whereas evidence has accumulated implicating archaea in periodontitis [20,21].

Archaea are characterized by their broad-spectrum resistance to antimicrobial agents [5]. Knowledge about their behaviour towards antimicrobials is needed in the perspective of their potential pathogenic role. Also, antimicrobial susceptibility patterns can be used to design protocols for the decontamination of complex microbiota to select archaea [22]. We herein review data regarding the antimicrobial susceptibility patterns of archaea, emphasizing the methanogens found in humans.

Testing the Susceptibility of Archaea

In vitro susceptibility testing

In liquid medium, an archaeal inoculum of 10% (v/v) of a stock solution is distributed in a series of tubes containing the antimicrobial (macrodilution method). The inoculum is determined by a 0.4 optical density at 580 nm corresponding to ($4.42^{12} \pm 1.84^{11}$) cells/mL. After incubation, the MIC is indicated by the first tube exhibiting no visible growth [5]. In solid medium, the antimicrobial is incorporated into agar poured into Petri dishes. The archaeal inocula are then spread over the surface of the agar. After incubation, the MIC is determined by the inhibition of growth on the medium containing the lowest concentration of the antimicrobial [23]. Agar dilution, performed with a range of concentrations in a geometric progression, is the reference method [24–26]. The density of the archaeal inoculum is paramount, and must be adjusted with a photometer. Inocula were transferred in each Petri dish, resulting in a final inoculum of 10^5 cells/mL [24,27]. The reliability of tests is influenced by many parameters that must be strictly controlled. The culture medium that allows growth of archaea does not contain antimicrobial inhibitors [28]. The concentration of calcium and magnesium should be monitored, as concentrations above 10 mM may inhibit the activity of certain antimicrobials acting on membranes [29]. Likewise, the pH influences the activity of several antimicrobials [28]. The temperature and delay of incubation must be fixed [30]. Using a susceptible organism as a positive control is mandatory [5].

Antimicrobials Acting on the Cell Wall

Cell-wall synthesis inhibitors

Bacterial cell walls contain peptidoglycan, with *N*-acetylmuramic acid being the molecular signature for the presence of peptidoglycan [31]. Archaea are considerably more diverse in the composition of the cell wall; they lack peptidoglycan in any form, but instead, proteins, glycoproteins and polysaccharides cover the outside of the cell membrane [32]. In any case, the functions of the cell wall remain the same: containing the cytoplasm, shaping the organism, and adapting to and interacting with the environment [33].

β-Lactams, glycopeptides, lipoglycopeptide and fosfomycin are the principal families of antimicrobials acting on the bacterial cell wall or bacterial cell-wall synthesis (Fig. 1). The β-lactams include many bactericidal molecules. Their common features are a β-lactam nucleus and a similar mode of action by inhibiting the final step of peptidoglycan synthesis [34]. Glycopeptides are huge molecules that cannot pass through the porins. Their spectrum of activity is limited to Gram-positive bacteria. Glycopeptides inhibit the synthesis of peptidoglycan in its final phase. The three-dimensional structure of these molecules covers the D-Ala-D-Ala of the pentapeptide-disaccharide, ready to be incorporated in the peptidoglycan, preventing the action of glycosyl transferases and transpeptidases, and blocking the elongation of peptidoglycan [35]. Fosfomycin acts at the earliest stage of peptidoglycan synthesis, and must enter the cell to be active [36].

Fosfomycin and antimicrobials directed against peptidoglycan biosynthesis have no growth-inhibitory effect against archaea with MICs of >50–100 mg/L [5,37]. The activity of these antimicrobials against *M. smithii* has been investigated with the reference strain DSMZ 861 (http://www.dsmz.de). The high level of resistance of this strain to β-lactams and

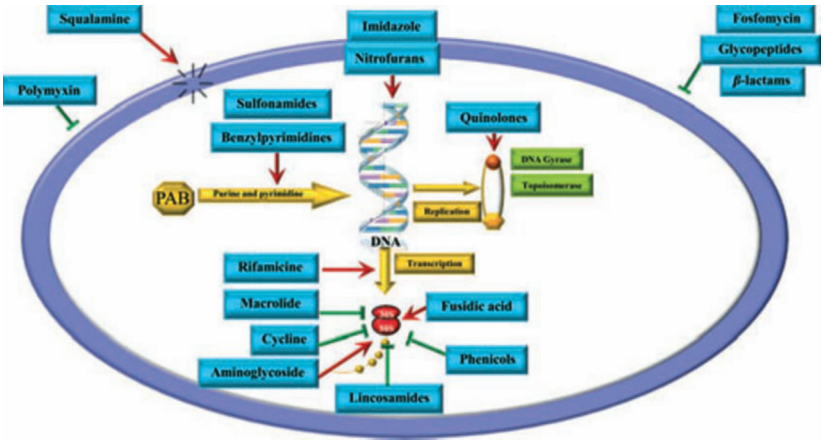

FIG. 1. Mode of action of antimicrobial agents against archaea. ⟶, Anti-archaeal activity observed. ⟞, No anti-archaeal activity observed. PAB, *p*-aminobenzoic acid.

glycopeptides was demonstrated by isolation procedures [22]. The resistance pattern of the faecal isolates agrees with the structural differences between bacteria and archaea [5], and this resistance is a natural attribute of these microorganisms [22]. Lack of peptidoglycan is the only documented mechanism of resistance. Indeed, different mesophilic methanogenic and extremely halophilic archaea containing pseudomurein or glycoprotein cell walls were tested for β-lactamase activity, with the chromogenic β-lactam nitrocefin as substrate. No β-lactamase activity was detected in any of the archaeal organisms [38]. This supports the view that β-lactamases are absent in archaea and are restricted to bacteria. Resistance to peptidoglycan inhibitors could be exploited for the selective isolation of archaea from complex microbiota. β-Lactams, glycopeptides and lipoglycopeptide are frequently used to isolate methanogens from human specimens containing a mixed microbiota, with the use of selective media to purify methanogen cultures [22].

Cell-wall-alterating antimicrobials

Polymyxin. Polymyxin B and polymyxin E (also known as colistin) are the two antimicrobial polypeptides used in clinical practice. They have a rapid bactericidal action by disrupting the lipidic components of membranes, including the lipopolysaccharide and the phospholipids [39]. Antimicrobial susceptibility testing to polymyxin E was performed on the haloalkaliphilic archaeon *Halalkalicoccus tibetensis* [40]. This strain was reported to be resistant to polymyxin E and several other antimicrobials, including penicillin, ampicillin, streptomycin, tetracycline, bacitracin, neomycin, and sulphafurazole, and to be susceptible to rifampicin and novobiocin, but no MIC was determined in these studies. The halophilic archaeon *Natronococcus amylolyticus* was found to be resistant to polymyxin B [41]. The susceptibility of halophilic archaea to this family of antimicrobial agents appears to be dependent on the strain tested, and may differ between closely related species [41,42]. Human methanogens were found to be susceptible to bacitracin, with MICs of <4 mg/L and <25 mg/L for *M. oralis* [5]. Such concentrations are achieved by topical utilization of bacitracin in oral formulations [43], suggesting that oral bacitracin could be used for the treatment of periodontitis where *M. oralis* has been implicated as a co-pathogen [20,21,44]. Susceptibility of human archaea to bacitracin has been exploited in the formulation of a medium for the selective isolation of *Streptococcus mutans* from human dental plaque [45].

Amphotericin B. In 2010, a study focused on halophilic archaea colonizing the human intestinal mucosa demonstrated the resistance of these microorganisms to this antifungal agent. Amphotericin B was therefore used in association with penicillin and erythromycin at 100 mg/L, to repress growth of salt-tolerant bacteria and fungi, with the aim of cultivating halophilic archaea from the human intestinal mucosa specimen [46]. No data were provided for the other archaeal families, including methanogens.

Squalamine and its derivatives. Squalamine is a potent, broad-spectrum antimicrobial molecule extracted from the livers of dogfish and other shark species [47]. It acts on Gram-negative bacteria by a mechanism similar to that of colistin, requiring interactions with the negatively charged phosphate groups of the bacterial outer membrane as the first step in a sequence of different events leading to the disruption of the membrane; squalamine exhibits a depolarizing effect on Gram-positive bacteria, resulting in rapid cell death [29].

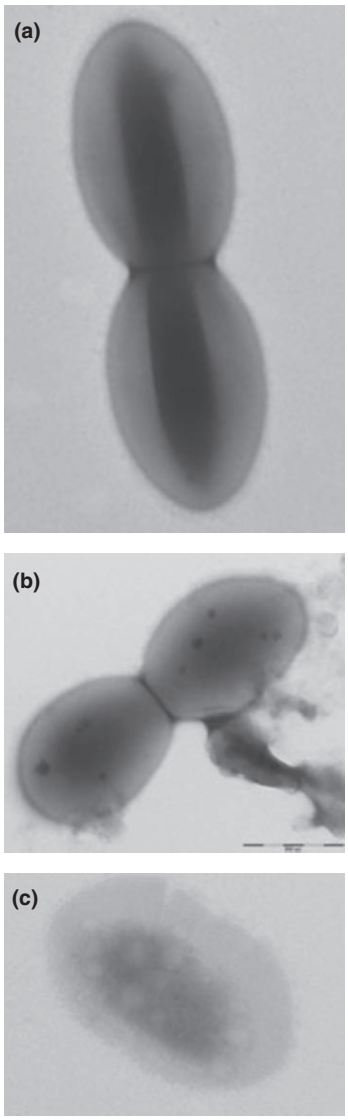

FIG. 2. Electronmicrographs showing the morphological effects of squalamine on the *Methanobrevibacter smithii* cell wall. (a) *M. smithii* without squalamine. (b) *M. smithii* + 1 μg/mL squalamine. (c) *M. smithii* after 12 h of incubation in a culture medium containing 1 μg/mL squalamine.

Squalamine is also effective against human methanogens, with an MIC of 1 mg/L [5] (S. Khelaifia and M. Drancourt, unpublished data). Our electron microscopy observations suggest that squalamine breaks the *M. smithii* cell wall, inducing cytoplasm leakage and cell death by a mechanism similar to that observed for Gram-negative bacteria (Fig. 2). Unpublished data from our laboratory indicate that human methanogens are susceptible to squalamine and some of its derivatives, with MICs between 0.1 and 1 mg/L (S. Khelaifia and M. Drancourt, unpublished data).

Antimicrobials interfering with DNA

DNA replication inhibition

DNA replication and transcription are targets for antimicrobials, including quinolones and novobiocin [48] (Fig. 1). Quinolones are synthetic antibacterial agents that were initially active against Gram-negative bacilli [48]. Quinolones enter cells by simple diffusion, and selectively inhibit DNA replication in bacteria and some archaea, acting at the level of supercoiling, which causes a reduction in the space occupied by DNA [48]. DNA gyrase is a topoisomerase involved in DNA supercoiling [49–51]. Quinolones form an irreversible ternary complex with the DNA gyrase, preventing gyrase activity and blocking replication.

Coumermycin, a quinolone derivative, was studied on several archaea at concentrations up to 200 mg/L. The results showed the susceptibility of halobacterial archaea to this compound, which also inhibits the growth of *Sulfolobus acidocaldarius* and members of the *Methanobacteriales*, *Methanococcales* and *Methanomicrobiales* [51]. The coumermycin MIC depended on the strain. Halophilic archaea were more susceptible, with an MIC of 5 mg/L, whereas thermophilic archaea exhibited an MIC of >200 mg/L [51]. Novobiocin is a bacteriostatic antimicrobial that is mainly active on Gram-positive bacteria by inhibiting DNA replication through preventing ATP binding to the DNA gyrase β-subunit [52]. Novobiocin was used to demonstrate the action of antimicrobial agents on the anaerobic digestion process [53]. The inhibitory action of novobiocin specifically affects the different populations involved in the final stage of anaerobic digestion. This hypothesis was confirmed by the lack of utilization of acetate and the partial degradation of propionate and butyrate [53].

Ansamycins form a family of secondary metabolites that show antimicrobial activity against many Gram-positive and some Gram-negative bacteria [54]. Moreover, ansamycins demonstrated antiviral activity towards bacteriophages and poxviruses [55]. Ansamycins inhibit the chaperone-mediated folding of Hsp90 substrates by blocking their ATP-dependent dissociation from Hsp90 [56]. The proeukaryote Hsp90 homologue HtpG is present in most bacterial species, but not in archaea [57].

Accordingly, the ansamycin rifampicin was shown to be ineffective on human archaea, with an MIC of >100 mg/L [5]. *H. tibetensis* [40] is a haloalkaliphilic archaeon previously reported to be resistant to resistant to polymyxin E and several other antimicrobials, including penicillin, ampicillin, streptomycin, tetracycline, bacitracin, neomycin, and sulphafurazole. This strain was reported to be susceptible

to rifampicin, but no MIC was determined in these studies.

DNA-altering antimicrobials: imidazole and derivatives

The spectrum of activity of imidazoles is limited to organisms whose metabolism is anaerobic or at least micro-aerophilic, such as *Helicobacter pylori* and *Gardnerella vaginalis* [58,59]. Indeed, imidazoles are prodrugs requiring partial reduction of the NO_2 group by anaerobic organisms [60,61]. Reduced imidazole derivatives are biologically active products that bind to DNA regions rich in adenine and thymine and cause oxidative cleavage of DNA stretches. Such DNA lesions are followed by the death of archaea and bacteria [59,61]. Metronidazole, an imidazole derivative, was initially shown to inhibit unidentified faecal methanogens with MICs between 0.5 and 64 mg/L [22]. It also showed *in vitro* activity against human methanogens, with an MIC of 1 mg/L [5]. It was, indeed, observed that treatment of the digestive tract with metronidazole in bone marrow transplant recipients eliminated detectable methanogens in stools: patients receiving metronidazole were negative for methanogen culture within the first week of therapy, and recolonization occurred within several weeks [62]. Gut decontamination with metronidazole suppressed or eliminated the methanogens, just as it did the anaerobic bacteria [63].

Nitrofurans are synthetic molecules used for treating intestinal tract infections (furazolidone and nifuroxazide) and urinary tract infections (nitrofurantoin and hydroxymethyl-nitrofurantoin) [64]. These molecules preferentially inhibit the synthesis of inducible enzymes by blocking the initiation of translation. The action of nitrofuran has implications for the regulation of gene expression in general [65]. Nitrofurans target DNA after reduction of the NO_2 group by aerobic bacterial nitroreductase [66]. Reduced derivatives break and induce mutations in DNA. Their effect is bacteriostatic or bactericidal, depending on the dose [67]. The anti-archaeal activity of nitrofurantoin was confirmed against the halophilic, aerobic archaea *Halobiforma haloterrestris* and *Halogeometricum borinquense*, without the determination of MICs [68].

DNA synthesis inhibitors: sulphonamides and benzylpyrimidines

Sulphonamides are synthetic molecules that are often combined with diaminopyridines (benzylpyrimidines) to increase their activity and to reduce the risk of resistance emergence. Sulphonamides are derivatives of *p*-aminobenzenesulphonic acid; the presence of a free amine and free sulphur radicals directly substituting benzene are essential for the antibacterial activity [69]. Sulphonamides and diaminopyridines inhibit the synthesis of folic acid, a key cofactor in the synthesis of purine and pyrimidine bases in prokaryotes [70,71], whereas eukaryotes directly assimilate folic acid from the diet. A detailed inhibition study of carbonic anhydrases belonging to the b and c carbonic anhydrase families from archaea with sulphonamides was presented for the first time in 2004 [72]. The two susceptibility carbonic anhydrases from *Methanosarcina thermophila* showed very different inhibitory properties than those from *Methanobacterium thermoautotrophicum*. The most potent inhibitors were sulphamic acid and acetazolamide, with MICs in the range 63–96 nM [72].

Protein Synthesis Inhibitors

The susceptibility of archaea to protein synthesis inhibitors has been determined by several groups [73,74] (Fig. 1). It has been known for some time that even closely related archaeal species are remarkably heterogeneous in their sensitivity to ribosome-targeted antimicrobials [75]. Many of the classical inhibitors of eubacterial 70S and eukaryotic 80S ribosomes do not inhibit the growth of these organisms even at high concentrations; inhibition is caused by only a few compounds that affect eubacterial and eukaryotic cells [5]. However, it is unclear whether this lack of susceptibility is caused by the impermeability of these organisms to most antimicrobials or by the lack of a ribosomal binding site [73]. The susceptibility of hyperthermophilic archaeal ribosomes to the inhibitory actions of all known classes of aminoglycoside antimicrobial has been tested on the hyperthermophilic *Aquifex pyrophilus*. *A. pyrophilus* ribosomes are susceptible to all tested aminoglycosides, including 2-deoxystreptamines, monosubstituted 2-deoxystreptamines, and streptidine [76]. The effect of selected aminoglycoside antimicrobials on the translational accuracy of poly(U) programmed ribosomes derived from the thermophilic archaea *Thermoptasma acidophilum*, *Sulfolobus solfataricus*, *Thermococcus celer* and *Desulfurococcus mobilis* showed that the four species investigated are markedly diverse in their response to the miscoding-inducing action of aminoglycoside antimicrobials [77]. A study of the susceptibility of human methanogens showed high *in vitro* resistance to gentamicin and streptomycin, with MICs of >100 mg/L [5].

Tetracyclines bind to the 30S subunit of the bacterial ribosome [78]. First-generation tetracyclines were obtained from chemical derivatives, doxycycline and minocycline, which have better bioavailability and increased tissue distribution, and a longer half-life for once-daily application [79]. Pactamycin was isolated from *Streptomyces pactum* as a potential

TABLE 1. Classification of antimicrobial agents according to their mode of action

Cell-wall synthesis inhibitors	DNA-interfering antimicrobials	Protein synthesis inhibitors	Cell-wall-alterating antimicrobials
(−) β-Lactams	(−) Ansamycins	(−) Tetracyclines	(−) Polymyxins
(−) Glycopeptide and lipoglycopeptide	(+) Quinolones	(−) Macrolides	(−) Amphotericin B
(−) Fosfomycin	(+) Novobiocin	(−) Lincosamides	(+) Squalamine
	(+) Imidazole	(−) Erythromycin	
	(+) Nitrofurans	(−) Phenicols	
	(+) Sulphonamides	(+) Aminoglycosides	
	(+) Benzylpyrimidines	(+) Fusidic acid	

(−), no anti-archeal activity observed; (+), anti-archeal activity observed.

new human antitumour drug, but is in fact a potent inhibitor of translation in eukaryotes, bacteria, and archaea [78]. Testing of the susceptibility of human archaea showed that all tested human methanogens were resistant to tetracycline at concentrations >100 mg/L [5].

Fusidic acid inhibits polypeptide chain elongation by binding to the ribosome elongation factor-G–GDP complex, thereby preventing its dissociation [80]. The interactions of fusidic acid with archaeal elongation factors were assayed by using poly(U) programmed cell-free systems under optimal culture conditions for polyphenylalanine synthesis. The effects of fusidic acid on the polyphenylalanine-synthesizing capacities of cell-free systems derived from representative members of the families *Methanobacteriaceae*, *Methanomicrobiaceae*, and *Methanococcaceae*, the reference eubacterial *Escherichia coli* and the eukaryotic *Saccharomyces cerevisiae* were investigated. The elongation factor-G equivalent factor (elongation factor-2) of all of the methanogens surveyed was systematically inhibited by fusidic acid within the same range of effective concentrations as that affecting the functionally homologous factors of *E. coli* and *S. cerevisiae*, at an MIC of 0.5 mg/L [81], supporting the hypothesis that archaea are susceptible to molecules that are also active against bacteria and eukaryotes [5].

Macrolides are active against Gram-positive and some Gram-negative bacteria [82] by inhibiting the elongation of the peptide chain after binding to the 50S subunit of bacterial ribosomes [82,83]. As β-lactams, macrolides are frequently used in association with other antimicrobial mixtures for laboratory decontamination to isolate methanogens from human specimens [22].

As previously described, erythromycin, an antimicrobial of the macrolide family, was used to cultivate halophilic archaea from human faeces [46]. These microorganisms were resistant to concentrations of approximately 100 mg/L [46].

Phenicols

Chloramphenicol, thiamphenicol and florphenicol bind preferentially to the A site at the 50S subunit [84]. The mechanism of action of chloramphenicol remains unclear, but these agents probably inhibit peptide binding and block chain elongation [85].

Archaea are generally less sensitive to phenicols; the growth of *Halobacterium halobium* and *Sulfolobus acidocaldarius* is inhibited at elevated concentrations of chloramphenicol, at MICs of ≥100 mg/L [86]. The *in vitro* susceptibility of human archaea to chloramphenicol is variable. *M. smithii*, *M. oralis* and *Methanomassiliicocus luminyensis* are resistant, with an MIC up to 25 mg/L, in contrast to *Methanosphaera stadtmanae*, which exhibits an MIC of 4 mg/L [5]. The *M. smithii*, *M. oralis* and *Methanomassiliicocus luminyensis* genomes encode a chloramphenicol *O*-acetyltransferase, an enzyme that inactivates chloramphenicol, but the gene for this is absent in the *Methanosphaera stadtmanae* genome [5].

Conclusions

This review of the data regarding the susceptibility of archaea to antimicrobial agents indicates that these organisms are broadly resistant to the antibiotics routinely used for the treatment of bacterial infections in humans (Table 1). However, archaea are members of microbial communities, and rely on bacterial metabolism for their own survival and multiplication. Therefore, the elimination of bacteria, including anaerobes, in these communities could result in the indirect, unexpected elimination of antibiotic-resistant archaea. If the role of archaea in human infection is further documented [19], then anti-archaeal compounds in addition to metronidazole and fusidic acid will be useful. Also, further studies should aim to test the effectiveness of oral compounds such as bacitracin against archaea implicated in periodontitis [20,21].

Transparency Declaration

The authors have no conflict of interest regarding this paper.

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