The antimicrobial resistance pattern of cultured human methanogens reflects the unique phylogenetic position of archaea

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Objectives: Methanogenic archaea are constant members of the human oral and digestive microbiota retrieved, in particular, from periodontitis lesions. The objective of the study was to determine their susceptibility to antimicrobials.

Methods: Using the macrodilution method in Hungate tubes with optical microscope observation combined with monitoring methane production, we determined the antibiotic resistance characteristics of eight methanogenic archaea.

Results: Methanobrevibacter smithii strains were resistant to ampicillin, streptomycin, gentamicin, rifampicin, ofloxacin, tetracycline and amphotericin B, with MICs ≥100 mg/L; these strains were also highly resistant to vancomycin (MIC ≥50 mg/L). They were moderately resistant to chloramphenicol (MIC ≤25 mg/L), and were susceptible to bacitracin (MIC ≤4 mg/L), metronidazole, ornidazole and squalamine (MIC ≤1 mg/L). The susceptibility of Methanosphaera stadtmanae was the same as M. smithii, except for chloramphenicol (MIC ≤4 mg/L), and Methanobrevibacter oralis yielded the same data as M. smithii, except for bacitracin (MIC ≤25 mg/L). The antibiotic susceptibility pattern of ‘Methanomassiliicoccus luminyensis’, which was recently isolated from human faeces, was identical to that of M. smithii.

Conclusions: Human methanogenic archaea are highly resistant to antibiotics, being susceptible only to molecules that are also effective against both bacteria and eukarya. Methanogenic archaea are good candidates to test for antimicrobial activity against members of this unique domain of life. Further studies to develop new molecules specifically targeting archaea as potential causes of infection are warranted.

Keywords: Methanobrevibacter smithii, Methanosphaera stadtmanae, Methanomassiliicoccus luminyensis, Methanobrevibacter oralis, methanogenic archaea, microbiota, antimicrobial agents, susceptibility testing, metronidazole, chloramphenicol, bacitracin, squalamine

Introduction

Bacteria and archaea have long been classified together as prokaryotes, but analysis of the ribosome by Carl Woese in 1974 revealed that they form two distinct domains.1 These data have since been confirmed by differences in the core genomes of bacteria, archaea and eukarya.2 Within archaea and bacteria, metabolic processes are different, and the cell walls of archaea are different from those of bacteria, thus explaining why some antibiotics effective against bacteria are not effective against archaea.3,4 The difficulty of cultivating methanogenic archaea has so far prevented a systematic evaluation of the antimicrobial agents that are active against both archaea and bacteria or eukarya. Only scarce data have been reported for Methanobrevibacter smithii (Mb. smithii) and Methanosphaera stadtmanae (Ms. stadtmanae); no systematic testing has been reported for Methanobrevibacter oralis (Mb. oralis), which has been isolated on media containing cefalotin, clindamycin, kanamycin and vancomycin,2 and no data have been published for ‘Methanomassiliicoccus luminyensis’ (Mm. luminyensis), a new archaeon that we recently isolated from a human stool sample (Table 1).5–11 Lastly, the fact that the archaea are a permanent feature of the human oral and digestive microbiota makes knowledge of the susceptibility of cultivable archaea to different antimicrobials...
### Table 1. Summary of previous works on archaea and antibiotics with clinical relevance; only includes reports specifying the MICs

<table>
<thead>
<tr>
<th>Organism</th>
<th>Phylum</th>
<th>Antibiotic, MIC (mg/L)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Methanococcus vannielii</em></td>
<td>Euryarchaeota</td>
<td>bacitracin (10) chloramphenicol (100) penicillin G (2000) vancomycin (500) gentamicin (&gt;500) kanamycin (&gt;500) neomycin (31) streptomycin (&gt;500) tobramycin (&gt;500) erythromycin (&gt;500) chloramphenicol (0.5) tetracycline (125) anisomycin (500) rifampicin (250) ampicillin (&gt;500) aureomycin (125)</td>
<td>33</td>
</tr>
<tr>
<td><em>Methanosarcina barkeri</em></td>
<td>Euryarchaeota</td>
<td>gentamicin (&gt;500) kanamycin (&gt;500) neomycin (2) streptomycin (&gt;500) tobramycin (&gt;500) erythromycin (&gt;500) chloramphenicol (8) tetracycline (31) anisomycin (125) rifampicin (500) ampicillin (&gt;500) aureomycin (31) novobiocin (200) ciprofloxacin (100)</td>
<td>6</td>
</tr>
<tr>
<td><em>Methanobrevibacter arborphilus</em></td>
<td>Euryarchaeota</td>
<td>gentamicin (250) kanamycin (500) neomycin (125) streptomycin (&gt;500) tobramycin (250) erythromycin (500) chloramphenicol (2) tetracycline (500) anisomycin (2) rifampicin (500) ampicillin (&gt;500) aureomycin (250)</td>
<td>6</td>
</tr>
<tr>
<td><em>Methanobacterium bryantii</em></td>
<td>Euryarchaeota</td>
<td>gentamicin (125) kanamycin (250) neomycin (125) streptomycin (&gt;500) tobramycin (125) erythromycin (&gt;500) chloramphenicol (2) tetracycline (250) anisomycin (2) rifampicin (500) ampicillin (&gt;500) aureomycin (250)</td>
<td>6</td>
</tr>
</tbody>
</table>
In this study, we assessed the effectiveness against cultured human methanogenic archaea of different molecules used against eukarya or bacteria. Interestingly, we found that only agents with activity against both eukarya and bacteria had activity against cultured human methanogenic archaea.

### Methods

#### Strains and culture

Mb. smithii ATCC 35061<sup>T</sup> DSMZ 861, Mb. smithii DSMZ 2374, Mb. smithii DSMZ 2375, Mb. smithii DSMZ 11975, Mb. oralis DSMZ 7256<sup>T</sup> and
Ms. stadtmanae ATCC 43021 T DSMZ 3091 were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), and were grown on media 119 and 322, depending on the strain (http://www.dsmz.de), at 37 °C in Hungate tubes (Dutsch, Issy-les-Moulineaux, France) under an H2/CO2 (80/20) atmosphere with agitation. ‘Mm. luminyensis’ CSUR P135T was isolated in our laboratory from human stool samples and was cultivated using Methanobrevibacter medium (medium 119; http://www.dsmz.de) modified by the addition of methanol under a hydrogen atmosphere.

### Antimicrobial susceptibility testing

Ampicillin, streptomycin, gentamicin, rifampicin, bacitracin, chloramphenicol, ofloxacin, tetracycline, amphotericin B and vancomycin were all obtained from Sigma (Saint Quentin Fallavier, France). Metronidazole and ornidazole were purchased from B. Braun Medical SAS (Boulogne, France). Squalamine was a gift from Jean-Michel Brunel, Pharmacy Faculty, Marseilles, France. A filtered aqueous solution of each compound was anaerobically added to Hungate tubes containing distilled water, which were sterilized by autoclaving at 121 °C for 15 min under an N2/CO2 (80/20) atmosphere. Susceptibility was determined by transferring 500 μL of an exponentially growing culture into 4.5 mL of fresh medium containing 1, 2, 4, 10, 25, 50 or 100 mg/L antibiotic and incubating at 37 °C with agitation; the growth of archaea was observed after 5 days of incubation. Control cultures were also incubated without antibiotics to provide a baseline for the behaviour of the strains. Growth was assessed by optical microscope observation and by parallel methane production measurement using a GC-8A gas chromatograph (Shimadzu, Champs-sur-Marne, France). Growth controls with known susceptibility patterns. Growth controls with antibiotics to provide a baseline for the behaviour of the strains. Growth was assessed by optical microscope observation and by parallel methane production measurement using a GC-8A gas chromatograph (Shimadzu, Champs-sur-Marne, France) equipped with a thermal conductivity detector and a Chromosorb WAW 80/100 mesh SP100 column (Alltech, Carquefou, France). N2 at a pressure of 100 kPa was used as the carrier gas. The detector and the injector temperatures were 150 °C, and the column temperature was 200 °C.

Verst to the antimicrobial activity of the tested antibiotics, the following control experiment was done. The culture media 119 and 322 (http://www.dsmz.de), and ‘Mm. luminyensis’ medium were supplemented with 1, 2, 4, 10, 25, 50 or 100 mg/L antimicrobial compound, and were incubated at 37 °C in H2/CO2 (80/20) atmosphere for 5 days. Before incubation and on each day after incubation started, their activity was tested in a disc plate bioassay using clinical isolates of *Escherichia coli* and *Staphylococcus aureus* with known susceptibility patterns. Growth controls with adapted media instead of antibiotic dilutions were introduced in all experiments and all tests were done in triplicate. The MIC was defined as the lowest antibiotic concentration that inhibited methane production. Detection of β-lactamase activity was done using the cefinase test (Becton Dickinson, Le Pont de Claix, France).

### Bioinformatic analyses

The *M. smithii* chloramphenicol O-acetyltransferase (Gene ID: 5215732) was checked against GenBank using BlastP. A phylogenetic tree was derived from sequence alignment using the maximum-likelihood algorithm in Mega (www.megasoftware.net).

### Results and discussion

The activity of the tested antibiotics when incubated at 37 °C under an H2/CO2 (80/20) atmosphere was confirmed by observing the growth inhibition of *E. coli* and *S. aureus*, which were used as controls, after 5 days of incubation. As for *M. smithii*, *Ms. stadtmanae*, *M. oralis* and ‘Mm. luminyensis’, all control cultures incubated without antibiotics grew as expected, with methane production starting on the third day.

The four *M. smithii* strains under study were resistant to the tested ampicillin, streptomycin, gentamicin, rifampicin, ofloxacin, tetracycline and amphotericin B (MICs ≥100 mg/L), and to vancomycin (MIC ≥50 mg/L). These strains were moderately resistant to chloramphenicol (MIC ≤25 mg/L), and were susceptible to bacitracin (MIC ≤4 mg/L), metronidazole, ornidazole and squalamine, a new potent antimicrobial molecule (MIC ≤1 mg/L) (Table 2). The antibiotic susceptibility pattern of ‘Mm. luminyensis’ was identical to that of *M. smithii*. *Ms. stadtmanae* exhibited the same resistance profile as *M. smithii*, except for susceptibility to chloramphenicol (MIC ≤4 mg/L). *M. oralis* yielded the same data as *M. smithii*, except that it was moderately susceptible to bacitracin (MIC ≤25 mg/L). No β-lactamase activity was detected in these organisms.

The data reported herein extend previous knowledge on the antimicrobial susceptibility patterns of human methanogenic archaea (Table 1). Despite the fact that archaea and bacteria are quite similar in size and shape, most archaea have no cell

### Table 2. MICs (mg/L) of 13 antimicrobial agents for four cultured human archaea

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th><em>M. smithii</em> ATCC 35061T</th>
<th><em>M. smithii</em> F1 strain DSMZ 2374</th>
<th><em>M. smithii</em> DSMZ 2375</th>
<th><em>M. smithii</em> B181 strain DSMZ 11975</th>
<th><em>M. oralis</em> DSMZ 7256</th>
<th><em>Ms. stadtmanae</em> ATCC 43021T</th>
<th>‘Mm. luminyensis’ CSUR P135T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>≥100</td>
<td>≥100</td>
<td>≥100</td>
<td>≥100</td>
<td>≥100</td>
<td>≥100</td>
<td>≥100</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>≥100</td>
<td>≥100</td>
<td>≥100</td>
<td>≥100</td>
<td>≥100</td>
<td>≥100</td>
<td>≥100</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>≥100</td>
<td>≥100</td>
<td>≥100</td>
<td>≥100</td>
<td>≥100</td>
<td>≥100</td>
<td>≥100</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>≥100</td>
<td>≥100</td>
<td>≥100</td>
<td>≥100</td>
<td>≥100</td>
<td>≥100</td>
<td>≥100</td>
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<tr>
<td>Rifampicin</td>
<td>≥100</td>
<td>≥100</td>
<td>≥100</td>
<td>≥100</td>
<td>≥100</td>
<td>≥100</td>
<td>≥100</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>≥100</td>
<td>≥100</td>
<td>≥100</td>
<td>≥100</td>
<td>≥100</td>
<td>≥100</td>
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<tr>
<td>Tetracycline</td>
<td>≥100</td>
<td>≥100</td>
<td>≥100</td>
<td>≥100</td>
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<td>≥100</td>
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<tr>
<td>Vancomycin</td>
<td>≥50</td>
<td>≥50</td>
<td>≥50</td>
<td>≥50</td>
<td>≥50</td>
<td>≥50</td>
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<tr>
<td>Metronidazole</td>
<td>≥1</td>
<td>≤1</td>
<td>≤1</td>
<td>≥50</td>
<td>≤1</td>
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<tr>
<td>Ornidazole</td>
<td>≥1</td>
<td>≤1</td>
<td>≤1</td>
<td>≥50</td>
<td>≤1</td>
<td>≤1</td>
<td>≤1</td>
</tr>
<tr>
<td>Bacitracin</td>
<td>≤4</td>
<td>≤4</td>
<td>≤4</td>
<td>≤4</td>
<td>≤4</td>
<td>≤4</td>
<td>≤4</td>
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<tr>
<td>Squalamine</td>
<td>≤1</td>
<td>≤1</td>
<td>≤1</td>
<td>≥50</td>
<td>≤1</td>
<td>≤1</td>
<td>≤1</td>
</tr>
</tbody>
</table>
wall and the cell wall structure of the Methanobacteria is uniquely composed of pseudomureins; also, the archaeal cell membrane is composed of glycerol-ether lipids. On the other hand, bacteria cell walls are mainly composed of peptidoglycans and their cell cytoplasmic membrane is composed of glycerol-ester lipids. Accordingly, the tested archaea were resistant to cell wall inhibitors, including peptidoglycan inhibitors, the resistance to which had been previously reported. The tested methanogens were also resistant to amphotericin B, in agreement with the absence of polynes in archaeal walls. Conversely, the tested archaea were susceptible to bacitracin, a previously reported inhibitor of pseudomurein-containing methanogens; bacitracin forms complexes with intermediates of the pseudomurein lipid cycles and prevents the dephosphorylation of polyisoprenoid pyrophosphate, which translocates building blocks of the cell wall across the inner membrane. It was previously established that bacitracin inhibits the growth of Mb. smithii and Ms. stadtmanae at 10 mg/L. Because of its

Figure 1. A phylogenetic tree (maximum-likelihood algorithm) based on the chloramphenicol O-acetyltransferase gene sequence of the five archaeal strains harbouring the gene (625 bp) and the corresponding region in eight bacteria having the enzyme with the higher sequence similarity (>90%) with Mb. smithii chloramphenicol O-acetyltransferase. The accession numbers (GI numbers for a complete genome) are indicated in the brackets. Only bootstraps of >90% are indicated. The scale bar represents a 2% nucleotide substitution rate.

Figure 2. Activity spectrum of 11 antimicrobial families against bacteria, eukarya and archaea, illustrating that the latter organisms are susceptible only to molecules also active against both bacteria and eukarya. A, amphotericin; B, rifampicin; C, quinolones; D, aminoglycosides; E, tetracyclines; F, chloramphenicol; G, glycopeptides; H, β-lactams; I, squalamine; J, azoles; and K, bacitracin. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.
systemic toxicity, bacitracin is usable only as a local antibiotic for potential decontamination of vancomycin-resistant Enterococcus faecium and for treating Clostridium difficile-induced diarrhoea. In addition, the tested methanogenic archaea were all susceptible to squalamine, a molecule exhibiting a depolarizing effect on Gram-positive bacteria, resulting in rapid cell death. This molecule also directly interacts with the negatively charged phosphate groups in the Gram-negative bacterial outer membrane, leading to its disruption.

We further observed that the tested methanogens were resistant to aminoglycosides and tetracyclines, two antibiotic families targeting the ribosome and interfering with the synthesis of proteins (Table 2). As for aminoglycosides, the Mb. smithii genome (NC_009515) and the Ms. stadtmanae genome (NC_007681) encode mutations in the rps12p and L6 genes that confer resistance to gentamicin and streptomycin, respectively, in bacteria. Chloramphenicol is another antibiotic that interferes with protein synthesis in the ribosome, and we observed that Mb. smithii, ‘Mm. luminyensis’ and Ms. oralis were resistant to chloramphenicol, in contrast to Ms. stadtmanae, using the criteria applied to anaerobic bacteria. In vitro susceptibility to chloramphenicol has been previously reported, with MICs in the same range as determined here, and we noticed for the first time that the Mb. smithii and the ‘Mm. luminyensis’ genome, but not the Ms. stadtmanae genome, encode a chloramphenicol O-acetyltransferase, an enzyme inactivating chloramphenicol. Our bioinformatics analyses indicated that, among archaea, this enzyme is only present in six methanogenic organisms (Figure 1) and is more closely related to the one found in Clostridium species with a sequence similarity of 96%–100% (Figure 1). Whether these data support a potential lateral gene transfer between bacteria and archaea warrants further study. These data agree with the previously reported corpus of data indicating the unique structure of the archaeal ribosome, as outlined by the initial 16S rDNA sequence-based differentiation of this domain from those of bacteria and eukarya. The tested methanogenic archaea were also resistant to antibiotics interfering with DNA replication and transcription, including rifampicin, as previously reported for Mb. smithii and Ms. stadtmanae (Tables 1 and 2). Rifampicin is a known inhibitor of the bacterial RNA polymerase and the archaeal RNA polymerase is structurally more closely related to the eukaryotic one. Mb. smithii, Ms. stadtmanae, Ms. oralis and ‘Mm. luminyensis’ were also resistant to ofloxacin, a fluoroquinolone inhibitor of bacterial DNA replication and transcription (Table 2). This result agrees with the previous observation of a ciprofloxacin MIC of ≥100 mg/L for seven different environmental archaea and of 15 mg/L for Natronobacterium gregoryi. As this type II DNA topoisomerase is present in archaean genomes, such high MICs are indicative of a very weak interaction between the archaean DNA gyrase-like enzyme and fluoroquinolones. Moreover, we observed that the Mb. smithii and Ms. stadtmanae genomes encode multidrug efflux systems, which could also contribute to the broad spectrum resistance of these organisms to antibiotics, including fluoroquinolones. On the other hand, all tested methanogenic archaea were susceptible to imidazoles, the reduced derivatives of which directly interact with and break nucleic acids. Metronidazole was previously shown to inhibit unidentified faecal methanogens. In the latter study, the MICs, which were between 0.5 and 64 mg/L, were below the faecal concentration of metronidazole achieved under therapeutic conditions, and the isolates were therefore all susceptible to metronidazole. It was indeed observed that metronidazole treatment of the digestive tract in bone marrow transplant recipients eliminated detectable methanogens in the stool.

Conclusions
In conclusion, we observed that the four tested methanogenic archaea were resistant to molecules that inhibit only bacteria or only eukarya, such as amphotericin B, but were susceptible to molecules that were effective against both bacteria and eukarya (Figure 2), including imidazoles and squalamine. Indeed, metronidazole and other azoles were found to be active against bacteria, in the case of anaerobes, and against eukarya, as in the case of Giardia lamblia, Entamoeba histolytica and Trichomonas vaginalis. Squalamine is a new potent antimicrobial agent reported to inhibit both Gram-positive and Gram-negative bacteria and fungi. Taking into account these results, the susceptibility of methanogenic archaea to antibiotics reflects the phylogenetic position of the archaea as a unique domain of life, different from that of bacteria, eukarya and large DNA viruses. Moreover, the antimicrobial susceptibility data reported herein may help in designing selective media for the isolation of new archaea from diverse environmental and host-associated microbiota. These data further point to the opportunity to develop new families of molecules for the specific inhibition of archaea, especially in light of the potential role of these organisms in animal and human infections.

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Transparency declarations
The co-authors declare that they are the co-inventors of a pending patent on the culture of ‘Mm. luminyensis’.

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3. De Rosa M, Gambacorta A, Giozzi A. Structure, biosynthesis, and antimicrobial susceptibility data reported herein may help in designing selective media for the isolation of new archaea from diverse environmental and host-associated microbiota. These data further point to the opportunity to develop new families of molecules for the specific inhibition of archaea, especially in light of the potential role of these organisms in animal and human infections.

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Archaea resistance pattern

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