Alamethicin Suppresses Methanogenesis and Promotes Acetogenesis in Bioelectrochemical Systems

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Microbial electrosynthesis (MES) systems with mixed cultures often generate a variety of gaseous and soluble chemicals. Methane is the primary end product in mixed-culture MES because it is the thermodynamically most favorable reduction product of CO₂. Here, we show that the peptaibol alamethicin selectively suppressed the growth of methanogens in mixed-culture MES systems, resulting in a shift of the solution and cathode communities to an acetate-producing system dominated by Sporomusa, a known acetogenic genus in MES systems. Archaea in the methane-producing control were dominated by Methanobrevibacter species, but no Archaea were detected in the alamethicin-treated reactors. No methane was detected in the mixed-culture reactors treated with alamethicin over 10 cycles (~3 days each). Instead, acetate was produced at an average rate of 115 nmol ml⁻¹ day⁻¹, similar to the rate reported previously for pure cultures of Sporomusa ovata on biocathodes. Mixed-culture control reactors without alamethicin generated methane at nearly 100% coulombic recovery, and no acedate was detected. These results show that alamethicin is effective for the suppression of methanogen growth in MES systems and that its use enables the production of industrially relevant organic compounds by the inhibition of methanogenesis.

Microbial electrosynthesis (MES) of organic products is a novel strategy in which microorganisms use electrons derived from an electrode to reduce carbon dioxide to organic chemicals. By using this technology, the greenhouse gas CO₂ can be converted into fuels (e.g., methane) or other useful organic commodities such as acetate (1, 2). When renewable energy from the sun, wind, or tides is used to drive CO₂ conversion, this process is also an attractive method for energy storage and distribution.

The production of methane from CO₂ reduction by microorganisms with electrons drawn from an electrode was reported for the first time by Cheng et al. (3). In this system, CO₂ was reduced to methane directly from current at a set potential of <−0.5 V (versus a standard hydrogen electrode [SHE]). However, methane is an inexpensive resource, and other products such as fatty acids or pharmaceutical precursors may be more desirable. It was previously shown that acetate can be produced on cathodes by pure (4) or mixed (5) cultures. In the latter, however, methane was always the main product, and acetate was produced in smaller amounts. In order to obtain a higher rate of acetate production, the addition of a methanogenic inhibitor was necessary (5, 6).

Two common inhibitors of methanogenesis are 2-bromoethanesulfonate (BES) and methyl fluoride. BES is an inhibitor of the methyl coenzyme M reductase (MCR) of methanogens (7), which is required for energy conservation in all known methanogenic archaea. BES competitively binds to the MCR enzyme to replace the methyl coenzyme M cofactor, which prevents coenzyme M recycling and therefore the capability to conserve energy. Methyl fluoride is frequently used to specifically inhibit acetoclastic methanogenesis, thus allowing the determination of the relative contribution of acetate versus H₂/CO₂ to total methane production in natural environments, but its mechanism is unknown (8). Both of these inhibitors suppress methanogenesis only temporarily, which can resume once the chemicals are removed from solution. They are not considered a viable strategy for long-term and large-scale MES systems because the amount added (up to 50 mM BES) can be toxic to other microorganisms, and the use of these chemicals at high concentrations can be uneconomical (9). Like BES and methyl fluoride, alamethicin is an inhibitor of methanogenesis, as demonstrated by a previous study on different peptide antibiotics (10). Its mechanism is the permeabilization of cell membranes, resulting in their depolarization, which prevents cells from building up a transmembrane gradient (11, 12). Unlike BES and methyl fluoride, alamethicin is a peptaibol, a type of polypeptide that contains the nonessential amino acid 2-aminoisobutyrate. This unusual amino acid induces helical peptide structures, making the peptide amphiphilic (13). Amphiphilic peptides are surfactants that reduce the interfacial tension between lipid layers and the surrounding aqueous phase, essentially disrupting the spherical structure of hydrophobic vesicles. Ultimately, alamethicin exposure leads to the disintegration of the cells via a germicidal soap effect, which is a different inhibitory mechanism than the specific inhibition of methanogens by BES.

Here, we investigated the effect of alamethicin on bioelectrical CO₂ reduction to methane or acetate in mixed-culture microbial electrolysis cells (MECs). Alamethicin was added at different concentrations (25, 50, or 100 μg ml⁻¹) into single- or double-chamber...
MECs. Methane production was compared by using single-chamber MECs, and the production of acetate was examined in two-chamber MECs with the addition amethicin or BES relative to controls (no inhibitors). The abundances and compositions of Bacteria and Archaea were also examined to better understand the effects of alamethicin on the microbial communities.

MATERIALS AND METHODS

Reactor configuration and operation. Single-chamber MECs were constructed by using clear glass serum bottles containing 5 ml solution with 3.7 ml headspace (14). The anodes were graphite plates with a size of 1 cm by 1.5 cm (0.32 cm thick) (GM-10; Graphite Store, Buffalo Grove, IL, USA), which had been polished by using sandpaper (grit type 400), and were rinsed at least 10 times with Milli-Q water. A fixed voltage of 0.7 V was applied between the anode and the cathode for the anodes (Ti) and cathodes (SS) were inserted through the rubber stopper. A fixed voltage of 0.7 V was applied between the anode and the cathode was −1 cm. Bottles were sealed by using thick butyl rubber stoppers (20-mm diameter) and aluminum crimp caps. The electrode wires for the anodes (Ti) and cathodes (SS) were inserted through the rubber stopper. A fixed voltage of 0.7 V was applied between the anode and the cathode with a power supply, and a 10-Ω resistor was connected between the positive lead of the power supply and the anode to measure the current by recording the voltage drop across the resistor using a multimeter (model 2700; Keithley Instruments Inc., Cleveland, OH, USA).

Reactors (duplicate) were inoculated by using 1 ml of primary clarifier effluent collected from The Pennsylvania State University wastewater treatment plant, and 4 ml of growth medium was sparged and maintained under a CO2-N2 (20%/80%) atmosphere. The growth medium (final pH of 6.8) contained 0.82 g/liter sodium acetate as the electron donor for anodic microorganisms. Other inorganic compounds were as follows (per liter): 0.6 g NaH2PO4, 1.5 g NaH2BO3, 0.1 g KCl, 2.5 g NaHCO3, 10 ml minerals (1.5 mg/liter nitritosarcinaeous, 3 mg/liter MgSO4 · 7H2O, 1 mg/liter NaCl, 0.5 mg/liter MnSO4 · H2O, 0.2 mg/liter NiCl2 · 6H2O, 0.1 mg/liter FeSO4 · 7H2O, 0.1 mg/liter NiCl2, 0.1 mg/liter CaCl2 · 2H2O, 0.1 mg/liter ZnSO4, 0.01 mg/liter CuSO4 · 5H2O, 0.01 mg/liter AlK[SO4]2, 0.01 mg/liter H2BO3, and 0.01 mg/liter Na2MoO4 · 2H2O), and 10 ml vitamins (10 mg/liter pyridoxine hydrochloride, 5 mg/liter thiamine hydrochloride, 5 mg/liter riboflavin, 5 mg/liter nicotinic acid, 5 mg/liter calcium pantothenate, 5 mg/liter vitamin B12, 5 mg/liter p-aminobenzoic acid, 5 mg/liter thiotic acid, 2 mg/liter biotin, and 2 mg/liter folic acid) (15). After one cycle, the solutions of the MECs were replaced with 5 ml fresh growth medium every cycle by using sterile syringes. The reactors were sparged with CO2-N2 (20%/80%) gas at the beginning of every cycle. For reactors with alamethicin (≥5%) (Cyanam Chemcial, Ann Arbor, MI, USA), solid alamethicin at a final concentration of either 25, 50, or 100 μg ml−1 was added to the growth medium at the beginning of cycle 2 and cycle 7.

In order to further analyze the effects of alamethicin on chemical product production, additional two-chamber reactors were used, with their construction as described previously, with a few modifications (16). Briefly, Pt wires were used as the water-splitting anodes to avoid acetate as the electron donor, and the cathodes were stainless steel mesh (mesh size, 50 by 50) (type 304; McMaster-Carr, Cleveland, OH, USA) with the same size was used as the cathode. The distance between the anode and the cathode was −1 cm. Bottles were sealed by using thick butyl rubber stoppers (20-mm diameter) and aluminum crimp caps. The electrode wires for the anodes (Ti) and cathodes (SS) were inserted through the rubber stopper. A fixed voltage of 0.7 V was applied between the anode and the cathode with a power supply, and a 10-Ω resistor was connected between the positive lead of the power supply and the anode to measure the current by recording the voltage drop across the resistor using a multimeter (model 2700; Keithley Instruments Inc., Cleveland, OH, USA).

Pyrosequencing and quantitative PCR (qPCR). DNA samples from the anode, cathode, and solution of single-chamber reactors were obtained under each operating condition. Half of the anodic biofilm (1 cm by 0.75 cm) was removed from the surface by using sterile pipette tips, 2 ml of the solution was sampled by using a sterile syringe, and half of the cathode (1 cm by 0.75 cm) was removed by using sterile scissors. DNA was extracted and purified by using a Power Soil DNA isolation kit (Mo-Bio Laboratories, Carlsbad, CA, USA) and stored at −20°C.

Extracted DNA was amplified for pyrosequencing using 16S rDNA gene-targeting forward and reverse fusion primers at the Research and Testing Laboratory (Lubbock, TX, USA). The forward primer was constructed (5′ to 3′) with the Roche A linker (CCATCTATCCCTGCTGCTTGCTCAGCACTGAC), an 8- to 10-base barcode, and the 27F primer for Bacteria (GAGTTTGTATCTGGACGCTCA) (17) or the 341F primer for Archaea (TAXGCTGGYGCASCAG) (18). The reverse fusion primer was constructed (5′ to 3′) with a biotin molecule, the Roche B linker (CCCCCTGTGTGCTCAGGACTGAC), and the 519R primer (GTNTTACNGCNGGCKGCTG) for Bacteria (19) or the 1000R primer (GARGGRWGTGCAGTGGG) for Archaea (18). Amplification products were then pooled, cleaned, and size selected according to Roche 454 protocols (454 Life Sciences, Branford, CT, USA). Sequencing was performed on a 454 GS-FLX sequencer (Roche Diagnostics Corporation, Indianapolis, IN, USA) by utilizing the Titanium sequencing kit (Roche) to generate ~500-bp sequence reads.

Raw sequences were preprocessed by using the mothur program before data analysis (20). First, low-quality sequences (i.e., those with mismatches to the forward primer [≥2 bp] or barcodes [≥1 bp], a homopolymer longer than 8 bp, or a sequence length shorter than 300 bp) were eliminated to minimize the effect of random sequencing errors. Second, primers and barcodes were trimmed, and the quality of unique sequences was evaluated via alignment with the SILVA reference data set (small-subunit version 119 [http://www.arb-silva.de/]). Third, sequences that were close to more abundant sequences (<2 mismatches per 100 bp) were preclustered to their nearest abundant sequences, and chimera sequences from all the unique sequences were removed by using the UCHIME chimera-checking algorithm (21). Finally, processed sequences were classi-
fied according to the SILVA version 119 small-subunit nonredundant reference database.

*Bacteria* and *Archaea* were quantified by using qPCR as described previously (22, 23). Methanogens were specifically quantified by targeting their *mcrA* gene (24). The detection limits for *Bacteria* were 10^6 copies per electrode and 10^5 copies per total solution (5 ml). For *Archaea*, they were 10^5 copies (electrode) and 10^4 copies (solution), and for *mcrA*, they were 10^7 copies (electrode) and 10^6 copies (solution). The detection limits were derived from the lowest measurable standard or the PCR water control (whichever was higher).

**Nucleotide sequence accession number.** Sequences were uploaded to the Sequence Read Archive (SRA) under BioProject accession number PRJNA262035.

**RESULTS**

**Chemical production.** Methane was not produced when alamethicin (Alameth.) was added to single-chamber MECs (Fig. 1). For the control reactors without alamethicin, methane was produced at a low rate until cycle 5. After that, methane was produced at an average rate of 3.2 ± 0.1 μmol ml^-1 day^-1 (cycles 5 to 10). The coulombic recovery of methane production was almost 100% for the control reactor. The coulombic recovery based on acetate production was ~35% for the reactor with alamethicin. The missing electrons were possibly converted to other organics such as formate and 2-oxobutyrate, as shown by others (4, 25). When BES was added to a two-chamber reactor, acetate was generated during all 3 cycles at an average rate of 120 ± 97 nmol ml^-1 day^-1. Methanogenesis was not completely inhibited by using BES, as methane was produced at an average rate of 40 ± 23 nmol ml^-1 day^-1 even with repeated dosing of additional BES (Fig. 3).

**Microbial community analysis.** Pyrosequencing was used to study the composition of the microbial communities (*Bacteria* and *Archaea*) in the single-chamber MECs on the cathode, on the reactors (no alamethicin). Control reactors produced mainly methane, at an average rate of 273 ± 62 nmol ml^-1 day^-1 (Fig. 2B). For the last cycle, the coulombic recovery of methane production was almost 100% for the control reactor. The same stoichiometry applies for hydrogenotrophic methanogenesis in the controls. Controls were never alamethicin amended. Batch cycles ended where the line interrupts. Duplicates are shown with the same symbols.

**FIG 1** Gas concentration of methane (dark gray) and hydrogen (light gray to white) in the headspaces of single-chamber MECs at the end of each cycle. For alamethicin (Alameth.) reactors, only hydrogen is shown because the concentration of methane was always below the detection limit of 0.1 μmol. Hydrogen concentrations are displayed as one-quarter of the measured concentration to adjust to the 1-to-4 stoichiometry of hydrogenotrophic methanogenesis. In control reactors without alamethicin, the concentration of hydrogen was always below the detection limit of 50 nmol and is therefore not shown. In alamethicin-amended reactors, the inhibitor was added at the beginning of cycle 2 and cycle 7. Error bars show standard deviations of the means of data from triplicate reactors.

**FIG 2** The reaction products hydrogen, methane, and acetate before (left of the dashed line) and after (right of the dashed line) alamethicin (Alameth.) addition in two-chamber MEC reactors. (A) Homocacetogenesis is compared to one-quarter hydrogen to account for the 1-to-4 stoichiometry. (B) The same stoichiometry applies for hydrogenotrophic methanogenesis in the controls. Controls were never alamethicin amended. Batch cycles ended where the line interrupts. Duplicates are shown with the same symbols.

**FIG 3** The reaction products methane and acetate in a two-chamber MEC reactor, which was inhibited with 1 mM BES at each data point. Conc., concentration.
anode, and in solution when 100 μg ml⁻¹ of alamethicin was used, compared to the control reactor (no alamethicin) (Fig. 4).

The addition of alamethicin altered the archaeal community (Fig. 4A). *Methanobrevibacter*, a group of hydrogenotrophic methanogens, was dominant (92% for the cathode and 87% for the anode) in the control reactor (no alamethicin), while *Methanobacterium* (65% for the cathode and 50% for the anode) and *Methanobrevibacter* shared the archaeal community of the reactor with alamethicin. However, quantitative analysis of the Archaea, and specifically methanogens, using qPCR indicated that the methanogens were present at levels below the detection limit of <10⁵ cells (<0.1% of all cells) and therefore were virtually absent in the reactor with alamethicin (Fig. 5). In the untreated controls, archaeal cell numbers reached 10⁸ per reactor, compared to 10¹⁰ bacterial cells (1% of all cells). The *mcrA* copy numbers were higher than those of *Archaea* across all control samples by a factor of ~10, which can be explained either by a dominance of *mcrA* genes over 16S rRNA genes in the methanogens or by differences between the 2 detection methods (*mcrA* assays used SYBR green, and 16S assays used TaqMan probes).

The bacterial community on the cathode and anode in the reactor with alamethicin added was similar to that in the control reactor (Fig. 4B). *Bacteria* most similar to *Sporomusa* dominated the cathode (68% for the reactor with alamethicin and 69% for the control reactor), and *Bacteria* most similar to *Geobacter* prevailed on the anode (56% for the reactor with alamethicin and 85% for the control reactor). However, the bacterial community for the solution in the reactor with alamethicin addition (50% *Sporomusa*) was different from that in the control reactor (40% both *Arcobacter* and *Bacillus* but only 7% *Sporomusa*). Additionally, the number of *Bacteria* in the reactor with alamethicin was reduced to 10⁸ cells per reactor, compared to the control reactor with 10¹⁰ bacterial cells (Fig. 5).
Alamethicin dosages as low as 25 μg ml⁻¹ (13 μM) were sufficient to suppress methanogenesis in both single-chamber and two-chamber MECs. In contrast, continuous BES addition was required at a concentration 2 orders of magnitude higher than that of alamethicin (1 mM or 211 μg ml⁻¹) to suppress, but not completely eliminate, methanogenesis. Because the growth of methanogens was inhibited, electrical current was directed to acetate, the next thermodynamically most favorable product (26). When either inhibitor (alamethicin or BES) was added, the two-chamber MECs produced acetate at similar rates (average rates of production of 115 nmol ml⁻¹ day⁻¹ with alamethicin and 120 nmol ml⁻¹ day⁻¹ with BES), showing that the performance was reproducible and reactor specific. The addition of alamethicin also resulted in acetate production comparable to the previously reported rate of 170 nmol ml⁻¹ day⁻¹ using a pure culture of Sporomusa ovata in MES reactors (5, 25).

The alamethicin-inhibited reactor did not produce any measurable amount of methane during the cycle in which it was added. Hydrogen was produced instead, and its absence in subsequent cycles indicated that it was used for acetate production. Unlike alamethicin, BES did not completely inhibit methanogens in any cycle, and methane was produced at rates only slightly lower than those in uninhibited reactors. The rates of methane production obtained here in the presence of BES were similar to those previously reported (25 ± 16 nmol ml⁻¹ day⁻¹ for inhibited reactors versus 31 ± 11 nmol ml⁻¹ day⁻¹ for uninhibited reactors; both 2σ error limits) (16). As a result, repeated addition of BES is required to suppress methanogenesis. Higher BES concentrations (>10 mM) than those used here could have more effectively suppressed methane production, but high concentrations of BES can also inhibit the growth of other microorganisms and would become a substrate for microorganisms that degrade BES (27). The cost of alamethicin is comparable to that of BES, taking into account the high concentration of BES and repeated additions needed, although the commercial price of alamethicin (~$175 per kg) on a mass basis was much higher than that of BES (~$0.05 per kg).

The addition of alamethicin to the single-chamber MECs at a concentration of 100 μg ml⁻¹ diminished the archaean population below the qPCR detection limit. The bacterial population on the anode was reduced by 1 order of magnitude upon alamethicin addition, but the number of Bacteria on the cathode remained relatively stable. The different effects can be well explained by differences in the cell walls of the different microorganisms. It is likely that Sporomusa was unaffected by the addition of alamethicin, as it dominated in the solution and on the cathode. Sporomusa is a Gram-positive bacterium, and its thick murine capsule is more difficult to penetrate than the thinner Gram-negative cell wall of Geoacter (anode) or the even thinner protein envelopes of Methanobacterium and Methanobrevibacter (cathode). Since Geoacter was dominant on the anode, the number of bacteria on the anode was reduced by 1 order of magnitude with the addition of alamethicin. On the cathode, however, the suppressed methanogens were simply replaced by a Sporomusa-dominated bacterial community.

Outcome. The addition of the peptidel alamethicin to solutions used in MECs can be used to inhibit methanogenesis, and it substantially alters the composition of the microbial communities in these bioelectrochemical reactors. Few methanogens were detected in the reactor following the addition of alamethicin, while acetogenic Sporomusa species remained dominant. Compared to other methanogenesis inhibitors like BES, alamethicin more effectively inactivated the methanogens. The concentrations of alamethicin used here were also 2 orders of magnitude lower than those of BES, making this peptide a highly effective inhibitor in bioelectrochemical systems where methane production is not desired.

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REFERENCES


