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 acetate 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 expensive 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 alamethicin 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), soaked for 30 min to remove debris, cleaned by soaking in 1 M HCl overnight, and rinsed three times with Milli-Q water. Stainless steel (SS) 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. A fixed voltage of 0.7 V was applied between the anode and the reference electrode (model RE-5B; BASi, West Lafayette, IN, USA) (3 cm by 0.75 cm) was removed from the surface by using sterile pipette tips, 2 cm by 2 cm projected area was used as the cathode to provide a sufficient surface for biofilm growth, and the anode was changed to a less expensive, high-surface-area carbon brush. Both of these materials have been shown to be useful for producing highly effective bioelectrode conditions (16). A cathode potential of ~0.6 V versus SHE was set after the reactors were inoculated with the effluent catholyte (10%, vol/vol) of an adapted methanogenic MEC culture used in a separate experiment (16). We previously observed that batch cycles in methanogenic MECs last 3 to 5 weeks (before the medium must be replaced), which is why we terminated the inhibited reactor after ~4 weeks.

**Chemical analysis.** Gas samples (250 μl) used for analysis by gas chromatography (GC) were taken from the headspace of the reactors with a 250-μl gas-tight syringe. The pressure of the headspace was balanced with atmospheric pressure by inserting a graduated syringe before sampling. The gas volume was determined by the headspace volume and the graduated syringe. Methane and hydrogen concentrations were measured using a gas chromatograph (model 310C; SRI Instruments, Torrance, CA, USA) equipped with a molecular sieve column (6 ft) at 80°C. Liquid samples (5 ml) were taken from the reactors and filtered through 0.45-μm-pore-diameter cellulose acetate membranes. The acetate concentration was measured using a high-performance liquid chromatograph (HPLC) (model LC-20AT; Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with an Allure Organic Acids 5-μm column and a diode array detector. The injection volume was 50 μl. The mobile phase was 50 mM KH₂PO₄ buffer (pH 2.31, adjusted by H₃PO₄), and the flow rate was 1.0 ml min⁻¹. The detector was set at 210 nm. Rates for methane and acetate production were calculated by using the linear regression across the entire batch cycle for each individual reactor.

**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 rRNA gene-targeting forward and reverse fusion primers at the Research and Testing Laboratory (Lubbock, TX, USA). The forward primer was constructed (5'-TAAACGACCAAGGTCGACT-3') with the Roche A linker (CCATCTATCTCCCTCGTTCTCAG), an 8- to 10-base barcode, and the 27F primer for Bacteria (GAGTTTGATCMTGGCTCAG) (17) or the 341F primer for Archaea (GAGGGTACCTGAGGTCACA) (18). The reverse fusion primer was constructed (5'-TACTTGGGTCGACT-3') with a biotin molecule, the Roche B linker (CCTATCCCTGTTGCTCGGACGCTCAG), and the 519R primer (GTNTTACNGCGGCGKCTG) for Bacteria (19) or the 1000R primer (GARGGWRGTGCATGATC) 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 preclustering to their nearest abundant sequences, and chimeric 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**, 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 from triplicate reactors.

**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 mmol 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 mmol ml^{-1} day^{-1}. Methanogenesis was not completely inhibited by using BES, as methane was produced at an average rate of 40 ± 23 mmol 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 mmol ml^{-1} day^{-1} (Fig. 2B). For the last cycle, 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 mmol ml^{-1} day^{-1}. Methanogenesis was not completely inhibited by using BES, as methane was produced at an average rate of 40 ± 23 mmol ml^{-1} day^{-1} even with repeated dosing of additional BES (Fig. 3).

**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 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) Homacetogenesis 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 10⁶ cells per reactor, compared to the control reactor with 10¹⁰ bacterial cells (Fig. 5).
DISCUSSION
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 methanogenesis 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). Methane was produced at rates only slightly lower than those in uninhibited reactors. The two-chamber reactors had about 2 orders of magnitude higher concentration of alamethicin used here were also 2 orders of magnitude lower than those previously reported 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 g) on a mass basis was much higher than that of BES (~$0.05 per g).

The addition of alamethicin to the single-chamber MECs at a concentration of 100 μg ml$^{-1}$ diminished the archaeal 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 murein capsule is more difficult to penetrate than the thinner Gram-negative cell wall of *Geo bacter* (anode) or the even thinner protein envelopes of *Methanobacterium* and *Methanobrevibacter* (cathode). Since *Geo bacter* 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.

**Outlook.** The addition of the peptoid 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


