ARTICLE

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Effects of the Antimicrobial Tylosin on the Microbial Community Structure of an Anaerobic Sequencing Batch Reactor

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ABSTRACT: The effects of the antimicrobial tylosin on a methanogenic microbial community were studied in a glucose-fed laboratory-scale anaerobic sequencing batch reactor (ASBR) exposed to stepwise increases of tylosin (0, 1.67, and 167 mg/L). The microbial community structure was determined using quantitative fluorescence in situ hybridization (FISH) and phylogenetic analyses of bacterial 16S ribosomal RNA (rRNA) gene clone libraries of biomass samples. During the periods without tylosin addition and with an influent tylosin concentration of 1.67 mg/L, 16S rRNA gene sequences related to *Syntrophobacter* were detected and the relative abundance of *Methanosaeta* species was high. During the highest tylosin dose of 167 mg/L, 16S rRNA gene sequences related to *Syntrophobacter* species were not detected and the relative abundance of *Methanosaeta* decreased considerably. Throughout the experimental period, Propionibacteriaceae and high GC Gram-positive bacteria were present, based on 16S rRNA gene sequences and FISH analyses, respectively. The accumulation of propionate and subsequent reactor failure after long-term exposure to tylosin are attributed to the direct inhibition of propionate-oxidizing syntrophic bacteria closely related to *Syntrophobacter* and the indirect inhibition of *Methanosaeta* by high propionate concentrations and low pH.

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KEYWORDS: anaerobic; antimicrobial; macrolide; methanogenesis; propionate; tylosin

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Introduction

The widespread use of antimicrobials has led to the detection of these chemical compounds in the environment (Koplin et al., 2002). Waste streams from hospitals, animal husbandry, and pharmaceutical production facilities contain elevated concentrations of antimicrobials (Campagnolo et al., 2002; Kummerer, 2001; Zilles et al., 2005) and are commonly treated in systems that rely on biological processes. Acceptable treatment performance has been reported in anaerobic lagoons treating manure with concentrations of antimicrobials that would inhibit sensitive microorganisms (Jindal et al., 2006; Zilles et al., 2005), but the mechanism(s) for maintaining biological activity in the presence of antimicrobials and the associated questions of how and when antimicrobial resistance develops are not well understood.

Tylosin, a commonly used veterinary antimicrobial, is a macrolide obtained from *Streptomyces* (McGuire et al., 1961). Macrolides inhibit protein synthesis by interacting with the 50S subunit of the bacterial ribosome. Although macrolides are not expected to target methanogenic archaea (Auerbach et al., 2004; Garza-Ramos et al., 2001), the inhibition of anaerobic bacteria may result in indirect adverse effects on methanogenesis. Most Gram-negative bacteria are resistant to macrolides due to relative impermeability of the cellular outer membrane and active drug efflux systems (Nikaido, 1996). Resistance to macrolides in other organisms may result from target modification, enhanced drug efflux, and drug inactivation. The most widespread mechanism of macrolide resistance is target modification mediated by erythromycin ribosome

methylase genes, which prevents macrolide, lincosamide, and type B streptogramin (MLS_B) antimicrobials from binding to 23S ribosomal RNA (rRNA) (Jensen et al., 1999; Weisblum, 1995).

Previous studies of macrolide inhibition in anaerobic systems provide contradictory results, ranging from negligible effects (Camprubi et al., 1988; Chelliapan et al., 2006; Masse et al., 2000; Poels et al., 1984; Stone et al., 2009) to substantial decreases in treatment performance (Amin et al., 2006; Loftin et al., 2005; Sanz et al., 1996; Shimada et al., 2008a). In anaerobic batch tests, the addition of tylosin has inhibited acetate, propionate, and butyrate uptake (Amin et al., 2006; Sanz et al., 1996; Shimada et al., 2008a). To better understand the effects of tylosin on anaerobic treatment performance, we investigated what steps of the anaerobic food web are affected by tylosin and whether anaerobic activity in the presence of tylosin is due to antimicrobial resistant populations with similar function as the sensitive organisms or to the diversion of the electron flow to alternate pathways of the anaerobic food web.

We previously evaluated the effects of tylosin on the treatment performance and on specific steps in the anaerobic food web of a glucose-fed anaerobic sequencing batch reactor (ASBR) (Shimada et al., 2008a). At an influent concentration of 1.67 mg/L, decreases in the rates of propionate uptake and methane production rates were observed without significant effects on chemical oxygen demand (COD) removal efficiency and total biogas production. At an influent concentration of 167 mg/L, a decrease in the glucose uptake rate, the accumulation of acetate and propionate, and a marked decrease of reactor performance were observed. The observed inhibition was speculated to be due to direct effects of tylosin on butyrate- and propionate-oxidizing syntrophic bacteria and indirect effects of volatile fatty acid (VFA) accumulation on aceticlastic methanogens. However, the performance data and batch tests did not determine whether or not changes in the microbial community structure were occurring in the reactor, and the impact of tylosin on the prevalence of macrolide resistance was not evaluated in our previous work.

The purpose of the current study was to evaluate the effect of tylosin on the microbial community structure and the prevalence of antimicrobial resistance using fluorescence in situ hybridization (FISH) and 16S rRNA gene-directed clone library analyses.

Materials and Methods

Laboratory-Scale ASBR

Detailed information on the reactor setup, operation, and performance has been reported (Shimada et al., 2008a). In brief, a jacketed bioreactor with a 5-L working volume was inoculated using granular sludge from an upflow anaerobic sludge blanket reactor treating brewery wastewater and operated as an ASBR with 24-h cycles. The reactor was operated at 35°C with a hydraulic retention time (HRT) of 1.67 days, a solids retention time of 80 days, and an organic loading rate of 3.5 kg COD/(m^3/day). During the feeding step, 70 mL of a concentrated influent solution containing glucose (234.4 g/L) was mixed with 2.93 L of buffered dilution water and supplemented with vitamins and trace elements. The influent tylosin concentration was increased in a stepwise manner (0 mg/L from days 0 to 749; 1.67 mg/L from days 750 to 944; and 167 mg/L from days 945 to 1,039), resulting in tylosin loading rates of 0, 1, and 100 mg/(L/day).

FISH

Sludge samples were collected from the middle level of the ASBR during mixing, fixed with 50% ethanol, and stored in phosphate-buffered saline (PBS)/ethanol (1:1) solution at −20°C. Fixed samples were dispersed using a Model 500 sonic dismembrator (Fisher Laboratory Equipment, Pittsburgh, PA) for 30 s (5 s pulses with 7.5 s intervals). The sonication time was optimized as recommended by Zhou et al. (2007). Dispersed samples were diluted with sterile PBS, filtered through 0.2-mm polycarbonate membranes (Poretics Corporation, Livermore, CA), and transferred to gelatin-coated slides (Amann et al., 1990). The slides were dehydrated in a graded ethanol series (50%, 80%, and 100%; 3 min each) and air dried.

FISH was performed as previously described (De los Reyes et al., 1997; Zhou et al., 2009) using the oligonucleotide probes and formamide concentrations listed in Table I. The cells were stained on the slide with 1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO) and citifluor (Marivac Limited, Halifax, NS, Canada) was used as antifading agent. The slides were observed under 630× magnification on a Zeiss Axiovert 100 inverted microscope (Carl Zeiss, Oberkochen, Germany) with appropriate filter sets and an exposure time of 1 s for FISH response. Images of

Table I. Oligonucleotide probes, sequences, target groups, and formamide stringency used in this study.

Probe	Sequence (5′–3′)	Target	Formamide (%)	Refs.
S-D-Bact-0338-a-A-18 (Bact0338)	GCTGCCTCCCGTAGGAGT	Most bacteria	25	Amann et al. (1990)
L-P-Grps-1901-a-A-18 (HGC69a)	TATAGTTACCACCGCCGT	*Actinobacteria*	25	Roller et al. (1994)
S-G-Clos-0129-a-A-15 (CLOSXIVa)	CTGTATGAGGCAGGT	Clostridia cluster XIVa	30	Weber et al. (2001)
L-*-Bact-2053-a-A-13 (MLS_B)	CTGCCTTTCTGGG	MLS_B sensitive bacteria	12.5	Zhou et al. (2009)
S-S-Arch-0915-a-A-20 (Arch0915)	GTGCTCCCCCGCCAATTCCT	Most archaea	20	Stahl and Amann (1991)
S-F-Msae-0825-a-A-23 (MX825)	TCGCACCGTGGCCGACACCTAGC	*Methanosaeta*	40	Raskin et al. (1994)
S-G-Msar-0821-a-A-21 (MS821)	CGCCATGCCTGACACCTAGCGAGC	*Methanosarcina*	50	Raskin et al. (1994)

duplicate wells (containing approximately 2,000 cells per well) were acquired from random locations using a Coolsnap color camera (Roper Scientific, Tucson, AZ). Automated image processing was performed using the software Visilog v6 (NOESIS, Gif sur Yvette, France) and classification was performed using fuzzy c-means clustering with manual quality control (Zhou et al., 2007).

MLS_B resistance was quantified indirectly through hybridization of an oligonucleotide probe (MLS_B in Table I) to unmethylated, MLS_B-sensitive cells as described by Zhou et al. (2009). The prevalence of ribosomal methylation and presumed MLS_B resistance was calculated by subtracting MLS_B probe-positive cells from the Bact0338 count and normalizing to the Bact0338 count.

Clone Library Construction

ASBR sludge samples were pelleted in 2-mL microcentrifuge tubes and stored at −80°C. DNA was extracted using a FastDNA SPIN Kit (Qbiogene, Inc., Irvine, CA) and quantified using a NanoDrop ND1000 (NanoDrop Technology, Wilmington, DE). DNA extracts were visualized using electrophoresis on a 0.8% agarose gel. PCR targeting the 16S rRNA gene of all bacteria was performed as described by Dojka et al. (2000) using primers 8f and 1492r (Richardson et al., 2002). Triplicate PCR products were pooled, purified by gel electrophoresis, and extracted using a MinElute Gel Extraction Kit (QIAGEN, Inc., Valencia, CA). Purified PCR products from Phase 1 (0 mg/L tylosin, day 743), Phase 2 (1.67 mg/L tylosin, days 841 and 939), and Phase 3 (167 mg/L tylosin, day 1,030) were used to create clone libraries using a TOPO4 TA Cloning Kit (Invitrogen, Carlsbad, CA). One hundred ninety-two clones from each library were sequenced at the Genome Sequencing Center at Washington University (St. Louis, MO).

Table II. Classification and the relative abundance (%) of the 16S rRNA gene sequences obtained from the reactor during different operational conditions.

	Tylosin concentration[a]			
Classification	0 mg/L	1.67 mg/L-A	1.67 mg/L-B	167 mg/L
Actinobacteria	26.3	34.9	6.7	69.4
Propionibacteriaceae	5.0	8.7	2.7	17.4
Nocardioidaceae	5.0	4.8	1.3	9.9
Bacteroidetes	20.2	22.2	30.7	2.5
Rikenellaceae	14.1	15.9	13.3	2.5
Prophyromonadaceae	1.0	n.d.	n.d.	n.d.
Candidate Division OP10	n.d.	0.8	n.d.	n.d.
Candidate Division TM7	6.1	1.6	n.d.	n.d.
Chloriflexi	8.1	12.7	n.d.	n.d.
Anaerolinaeceae	1.0	0.8	n.d.	n.d.
Caldilineacea	7.1	8.7	n.d.	n.d.
Firmicutes	23.2	11.1	13.3	14.9
Insertae Sedis XV	3.0	4.0	n.d.	n.d.
Ruminococcaceae	1.0	1.6	2.7	n.d.
Clostridiaceae	8.1	n.d.	n.d.	n.d.
Veillonellaceae	9.1	4.0	9.3	2.5
Peptococcaceae	n.d.	n.d.	1.3	n.d.
Enterococcaceae	n.d.	n.d.	n.d.	12.4
Nitrospira	2.0	4.0	44.0	5.8
Nitrospiraceae	2.0	4.0	44.0	5.8
Proteobacteria	12.1	12.7	5.3	5.8
Enterobacteriaceae	n.d.	n.d.	n.d.	3.3
Syntrophobacteraceae	8.1	9.5	4.0	n.d.
Syntrophobacter	7.1	7.9	2.7	n.d.
Syntrophaceae	n.d.	1.6	n.d.	n.d.
Methyloccystaceae	n.d.	n.d.	n.d.	0.8
Desulfovibrionaceae	n.d.	n.d.	1.3	n.d.
Vibrionaceae	n.d.	n.d.	n.d.	1.6
Spirochaetes	2.0	0.8	n.d.	1.6
Spirochaetaceae	2.0	0.8	n.d.	1.6
Total identified clones	99	126	75	121

n.d., not detected.
[a]The four samples were collected on days 743, 841, 939, and 1,030, respectively.

Phylogenetic Analysis

Sequences obtained from the forward (T3) and reverse (T7) sequencing primers were separately uploaded to the Ribosomal Database Project (RDP) II release 9.5 using the Pipeline function (Cole et al., 2007). Because of the lack of overlap between the sequences retrieved with the two primers, only the sequences from the T7 primer were used for the phylogenetic analysis (Table II). Even among T7 initiated sequences, 40, 18, 52, and 51 sequences of the four libraries could not be aligned properly in RDP Pipeline. Successfully aligned sequences were further classified using the default 80% confidence threshold in RDP. Among them, a number of clones were classified as unidentified bacteria (53, 46, 65, and 20 clones for days 743, 841, 939, and 1,030, respectively).

Phylogenetic trees were built for clones related to the phyla Actinobacteria, Firmicutes, and Proteobacteria. Because there was no overlap between sequences obtained from the two primers, only the sequences originally amplified from the primer 1492r were used to build the phylogenetic trees. Clone sequences and the corresponding reference sequences were aligned using ClustalW (Chenna et al., 2003). All of the sequences passed the anomaly check in the program Mallard (Ashelford et al., 2006) and were used to constructed phylogenetic trees using the neighbor-joining algorithm in MEGA 4.0 (Tamura et al., 2007). Robustness was tested by bootstrap resampling from 1,000 replicates. All positions containing gaps and missing data were eliminated from the dataset. There are 245, 351, and 358 positions in the final datasets of Actinobacteria, Proteobacteria, and Firmicutes, respectively. Clones with sequence similarity higher than 95% were grouped into operational taxonomic units (OTU) (Fields et al., 2005). The software BioEdit was used to edit sequences during the phylogenetic analysis (Hall, 1999). The clones are available at GenBank with accession numbers GQ853694–GQ853879.

Mathematical Model

The ASBR was modeled as described previously (Shimada et al., 2008b). In brief, the IWA Anaerobic Digestion No. 1 (Batstone et al., 2002) was modified to include microbial storage and tylosin inhibition. Tylosin inhibition was included by increasing the decay rate of propionate oxidizing syntrophic bacteria. The model was implemented in the software Aquasim 2.1e (Reichert, 1994).

The tylosin inhibition constant and the Monod parameters for propionate and acetate uptake were estimated based on measured concentrations throughout ASBR 24-h cycles. Parameter estimation was performed using a weighted least squares method. ADM1 recommended values (Batstone et al., 2002) were used for the Monod parameters of growth of all microbial populations and uptake of the other soluble substrates.

Results and Discussion

Detailed information on the reactor performance has been reported previously (Shimada et al., 2008a). During the periods with no tylosin addition and 1.67 mg/L tylosin (days 600–944), the reactor showed excellent biogas production (10.4 L/day) and COD removal (99.5%) and low effluent VFA concentrations (8.9 mg/L as acetate). The treatment performance deteriorated substantially after increasing the tylosin feed to 167 mg/L (days 945–1,050) with biogas production of 2.9 ± 0.5 L/day, COD removal of 7.0%, and effluent VFA concentrations of up to 3,400 mg/L as acetate.

Domain-Level Community Structure

The abundance of bacteria and archaea were quantified using FISH (Fig. 1a). Prior to tylosin addition, bacteria represented $35.7 \pm 1.1\%$ (mean $\pm$ standard error) of the DAPI stained cells. Hybridization to the general bacterial probe (Bact0338) remained stable during the first 50 days of Phase 2 and then decreased to $23.1 \pm 0.7\%$. The increase of the tylosin influent concentration to 167 mg/L was followed by an increase in the relative level of bacteria ($40.3 \pm 3.6\%$). Hybridization to the general archaeal probe (Arch0915) began at $18.6 \pm 0.5\%$ but decreased to $12.7 \pm 1.1\%$ prior to tylosin addition. The levels then remained relatively stable throughout reactor operation with averages of $14.3 \pm 0.5\%$ during 1.67 mg/L tylosin addition and $13.5 \pm 0.9\%$ during 167 mg/L tylosin addition.

Together, hybridization to the general bacterial and archaeal probes accounted for $51.2 \pm 2.9\%$, $43.0 \pm 3.0\%$, and $53.8 \pm 6.6\%$ of the DAPI stained cells for Phase 1, Phase 2, and Phase 3, respectively. A protocol using paraformaldehyde as fixative (De los Reyes et al., 1997) was also tested but did not increase the total number of cells hybridized to the Bact0338 and Arch0915 probes. The relatively low number of hybridized cells may be due to the presence of inactive cells (i.e., low ribosome content) inside the granules. Large zones of DAPI stained cells without hybridization to general microbial probes have been observed in anaerobic granules previously and were attributed to substrate limitations at the center and low pH close to the surface of the granules (Batstone et al., 2004). Alternatively, it may reflect incomplete coverage of this microbial community by the Bact0338 probe, as a mixture of probes has been recommended to obtain a more complete bacterial count (Daims et al., 1999).

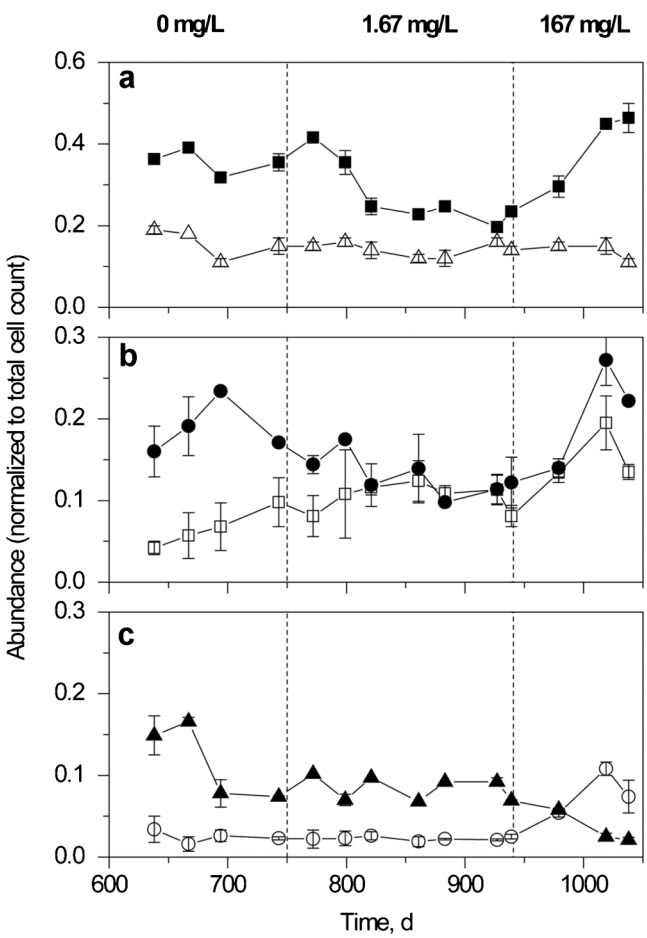

Figure 1. Fraction of microbial cells that hybridized to oligonucleotide probes targeting all bacteria (■) and all archaea (Δ) (**panel a**), high GC bacteria (●) and clostridia cluster XIV (□) (**panel b**), and *Methanosaeta* (▲) and *Methanosarcina* (○) (**panel c**) in ASBR biomass. The results were normalized to total cell counts with DAPI. Data points and error bars represent mean values and half ranges of duplicate wells, respectively. Numbers above the figure indicate influent tylosin concentrations.

Due to the relatively low percentage of hybridized cells, the results were normalized to the total number of cells that hybridized to the oligonucleotide probes Bact0338 and Arch0915 for comparison with literature values (Fig. 2a). Throughout the experimental period, the relative levels of bacteria and archaea (normalized to Bact0338 and Arch0915) were within the broad range reported in previous studies. Liu et al. (2002) reported that bacterial and archaeal cells comprised 40.8% and 62.9%, respectively, in granular

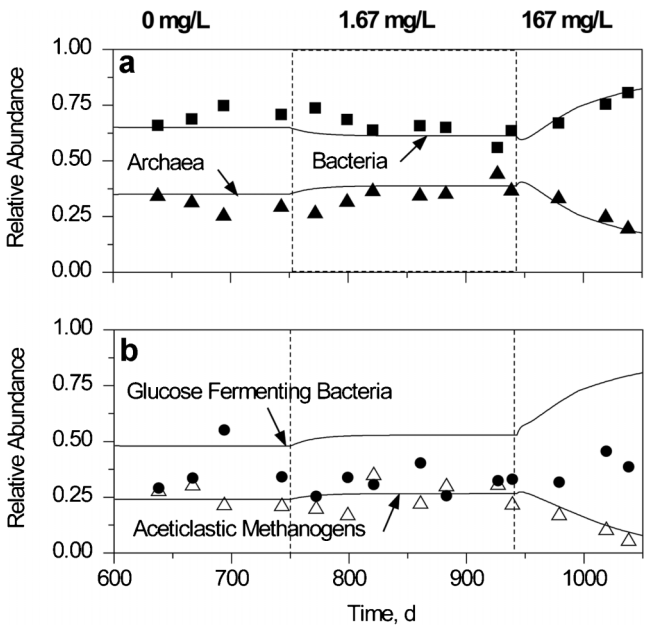

Figure 2. Modeled (–) and measured bacteria (■), archaea (▲), high GC bacteria (●), and aceticlastic methanogens (*Methanosarcina* + *Methanosaeta*, Δ) relative abundance in the ASBR throughout the experimental period. FISH data were normalized to total hybridization (sum of Bact0338 and Arch0915). Numbers above the figure indicate influent tylosin concentrations.

biomass samples from a UASB reactor treating brewery wastewater, based on membrane hybridizations. Zheng et al. (2006) reported that methanogenic archaea comprised 40% of the microbial community in a glucose-fed reactor, also based on membrane hybridizations. Sekiguchi et al. (1998) analyzed the phylogenetic diversity of granules from a mesophilic reactor fed with sucrose, acetate, and propionate and reported that 81% of the clones were affiliated with bacteria and 19% with archaea.

Bacterial Community Structure

The bacterial community structure was monitored throughout the reactor operation at a coarse level of resolution using FISH (Fig. 1b). Most of the bacteria (55–100%) were Gram-positive and belonged to the phyla Actinobacteria (HGC probe) and Firmicutes (ClosXIVa probe). The abundance of Actinobacteria (normalized to DAPI) was $19.2 \pm 1.5\%$, $11.8 \pm 1.0\%$, and $24.7 \pm 2.5\%$ during Phase 1, Phase 2 (days 799–944), and Phase 3 (days 1,009–1,039), respectively. *Clostridium* cluster XIVa increased ($4.2 \pm 0.8\%$ to $9.8 \pm 3.0\%$) throughout Phase 1 and remained stable ($10.4 \pm 0.7\%$) throughout Phase 2. During Phase 3, the clostridia increased to $15.5 \pm 1.6\%$. These levels of clostridia were considerably lower than those reported by Liu et al. (2002), where *Clostridium* spp. accounted for 66% of the total bacterial rRNA. At this coarse level of resolution, a clear impact of tylosin on the microbial community structure was not observed, despite changes in reactor performance.

Archaeal Community Structure

The archaeal community structure was also monitored throughout the reactor operation at a coarse level of resolution using FISH (Fig. 1c). The archaea were largely aceticlastic methanogens of the genera *Methanosaeta* and *Methanosarcina* (80–100%). The relative abundance of *Methanosaeta* prior to the addition of 167 mg/L tylosin (59% of total archaea) was comparable to or higher than previous studies (Liu et al., 2002; Zheng et al., 2006). The average abundance of *Methanosaeta* (normalized to DAPI) began at $15.7 \pm 0.8\%$ but dropped to $7.6 \pm 0.5\%$ prior to addition of tylosin and remained relatively stable during addition of 1.67 mg/L tylosin at $8.4 \pm 0.5\%$. However, the accumulation of propionate and acetate and decreasing methane production that occurred during the addition of 167 mg/L tylosin corresponded with a decrease in the *Methanosaeta* to $3.4 \pm 0.7\%$ and an increase in *Methanosarcina* from $2.3 \pm 0.3\%$ to $7.9 \pm 1.1\%$.

Tylosin is not expected to target archaea, so this shift in the methanogenic community is presumed to be an indirect effect, perhaps a pH effect. The accumulation of propionate and acetate caused the pH to drop to 6.0, below the reported range for growth of *Methanosaeta* of 6.8–8.2 (Huser et al., 1982) but within the broader reported pH range of 5.5–8.0 for *Methanosarcina* (Liu et al., 1985). Additional factors contributing to the shift could include the competitive advantage of *Methanosarcina* over *Methanosaeta* at higher acetate concentrations (Jetten et al., 1992), and *Methanosarcina's* ability to use a broad range of substrates (acetate, hydrogen and CO_2, methanol, and methylamines). Further acidification of the reactor resulted in pH levels of 5.2–5.5, below the optimum for *Methanosarcina*, and the complete inhibition of acetate uptake (Shimada et al., 2008a).

Some methane production was observed even after acetate uptake ceased. The modeled hydrogen production (Shimada et al., 2008b) was sufficient to produce 8% methane in the biogas, which corresponded to the measured levels, suggesting that the remaining methane production was due to the utilization of hydrogen.

Phylogenetic Analysis

To link specific bacterial populations with each stage of the anaerobic food web in the ASBR, clone libraries targeting the bacterial 16S rRNA genes were generated from four biomass samples collected over the three different phases of reactor operation (Table II). With the exception of the second sample collected during 1.67 mg/L tylosin addition (day 939), Actinobacteria was the most abundant phylum recovered from the ASBR. The phyla Bacteroidetes, Firmicutes, Nitrospira, and Proteobacteria were also

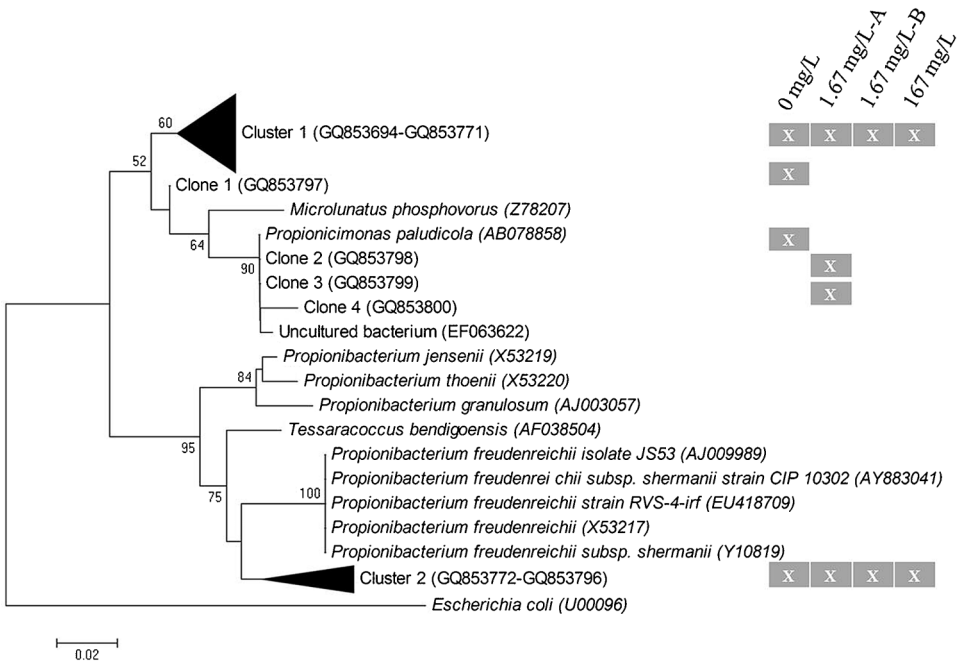

Figure 3. Phylogenetic tree showing the relationship among 1492R-derived sequences affiliated with the phylum Actinobacteria. The scale bar represents 0.02 changes per nucleotide, and the shaded boxes represent presence in the specified clone library.

frequently detected. The addition of 1.67 mg/L of tylosin corresponded with a considerable decrease in the recovery of sequences from the Firmicutes (23.2–11.1%) and increases in sequences related to Actinobacteria (26.3–34.9%) and Chloriflexi (8.1–12.7%). Following the addition of 167 mg/L tylosin, the recovery of sequences related to Actinobacteria increased to 69.4% while those related to Bacteroidetes dropped to 2.5%. The dynamics in the recovery of sequences related to Actinobacteria were not observed in the FISH results for the HGC probes, suggesting that any changes in their abundance in the reactor were contained within the HGC group. In general, less diversity was observed following the addition of tylosin.

Among the sequences affiliated to Actinobacteria, several were clustered with the genera *Propionibacterium* and *Propionicimonas* (1.0%, 1.6%, 0.0%, and 0.8% of Actinobacteria for days 743, 841, 939, and 1,030, respectively). These genera contain fermentative bacteria capable of converting glucose to propionate and acetate and were likely performing that function in our glucose-fed reactor. These results confirmed previous work in which, based on high levels of propionate production and transient accumulation of trehalose within the daily cycle, we suggested the presence of glucose fermenting propionibacteria in the ASBR (Shimada et al., 2007). The two major clusters in the phylogenetic tree in Figure 3 include Actinobacteria-affiliated clones from all of the four clone libraries, suggesting that the composition of the Actinobacteria population was relatively stable during the various phases of the reactor operation. With respect to tylosin resistance, minimum inhibitory concentrations (MIC) of less than 2 mg/L tylosin have been reported for sensitive strains of *Propionibacterium granulosum*, *Propionibacterium acnes*, and *Propionibacterium avidum* (Ross et al., 2002), while macrolide resistant strains were not inhibited at concentrations up to 512 mg/L tylosin (Ross et al., 2002). Our performance results suggest that the populations of Propionibacteriaceae in this reactor were resistant to tylosin.

Propionate uptake has been reported in both Gram-negative (*Syntrophobacter* and *Smithella*) and Gram-positive (*Desulfotomaculum* and *Pelotomaculum*) organisms (Boone and Bryant, 1980; de Bok et al., 2001; Imachi et al., 2002; Nilsen et al., 1996; Plugge et al., 2002). Because Proteobacteria-related clones from days 743, 841, and 939 were closely related to the genus *Syntrophobacter* (Fig. 4 and Table II) and no clones related to Gram-positive propionate utilizers were detected in the clone libraries, propionate uptake in the reactor was attributed to *Syntrophobacter*. This hypothesis is supported by the results from day 1,030, when propionate accumulated in the reactor and no clones related to *Syntrophobacter* were recovered (Table II). However, this is surprising in light of the performance results. Most Gram-negative bacteria are resistant to macrolides due to the relative impermeability of the cellular outer membrane, which functions together with active drug efflux systems (Nikaido, 1996), so if the Gram-negative *Syntrophobacter* were responsible for propionate uptake then neither the inhibition of propionate uptake observed while 1.67 mg/L tylosin was being fed nor the observed accumulation of

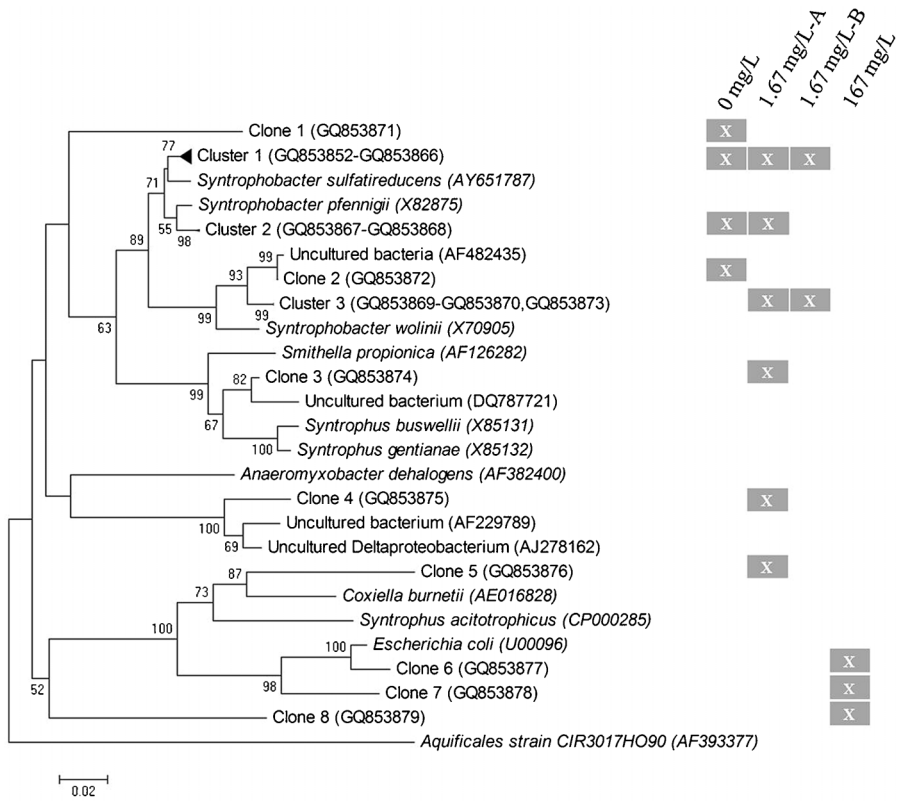

Figure 4. Phylogenetic tree showing the relationship among 1492R-derived sequences affiliated with the phylum Proteobacteria. The scale bar represents 0.02 changes per nucleotide, and the shaded boxes represent presence in the specified clone library.

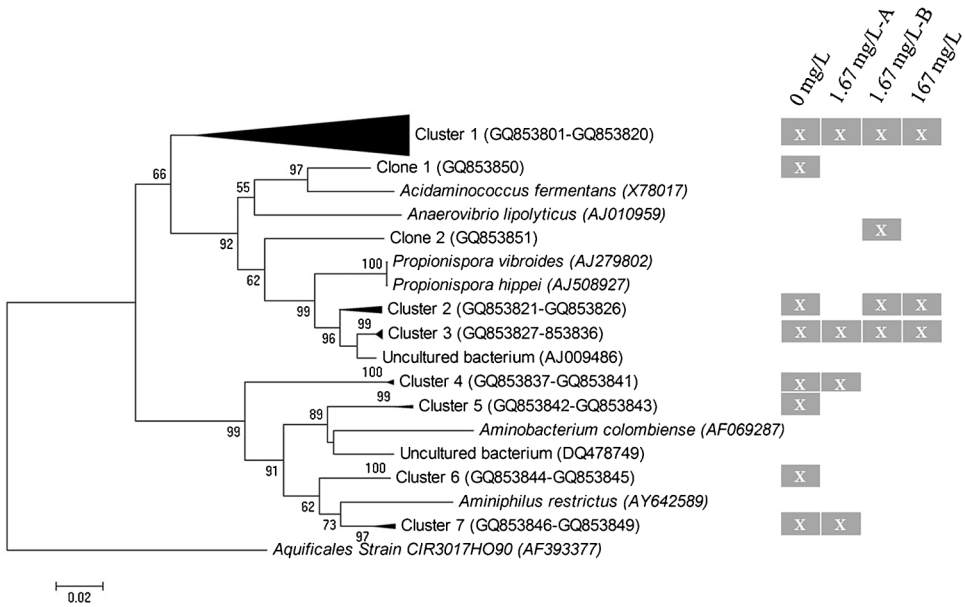

Figure 5. Phylogenetic tree showing the relationship among 1492R-derived sequences affiliated with the phylum Firmicutes. The scale bar represents 0.02 changes per nucleotide, and the shaded boxes represent presence in the specified clone library.

propionate while 167 mg/L tylosin was being fed would be expected. We were unable to find any reports on the effects of macrolides on *Syntrophobacter*, but macrolides have been used to treat infections caused by another bacterium of the order Desulfovibrionales, *Lawsonia intracellularis* (McOrist et al., 1997), so it is reasonable to postulate that *Syntrophobacter* are also sensitive to macrolides.

The accumulation of acetate when 167 mg/L tylosin was fed would be expected to create conditions for the growth of homoacetogenic bacteria. However, batch tests performed with ASBR biomass at neutral pH indicated that acetate uptake was completely inhibited during this period (Shimada et al., 2008a). Furthermore, the level of clones related to *Clostridium* was below detection in all of the samples except for day 743, when 5% of the clones were related to this genus. These results may suggest that tylosin inhibited the homoacetogenic bacteria, and are consistent with work by Amin et al. (2006), in which the partial inhibition of acetate uptake was attributed to direct effects of macrolides (i.e., erythromycin) on homoacetogenic bacteria.

MLS_B Resistance

The ASBR performance deteriorated during the addition of 167 mg/L tylosin and did not recover during the operational period considered here, so based on the performance data no increase in tylosin resistance was predicted. The FISH analysis was consistent with the performance-based prediction (Fig. 6). Specifically, a slight decrease in the prevalence of cells that hybridized to the bacterial probe but not the MLS_B probe (presumed MLS_B resistant bacteria) occurred after tylosin addition, from $56.2 \pm 3.9\%$ in the absence of tylosin to $38.5 \pm 3.2\%$ and $39.3 \pm 5.9\%$ under low and high tylosin dosing, respectively (Fig. 6). Angenent et al. (2008) reported an increase in MLS_B resistance in an ASBR

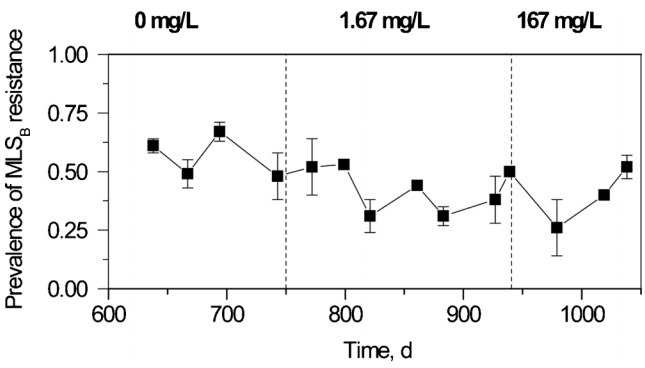

Figure 6. Prevalence of MLS_B resistance throughout the operational period. Cells were classified as MLS_B resistant if they hybridized to the Bact0338 probe but not to the MLS_B probe, as the same mismatch or site-specific methylation that results in MLS_B resistance prevents hybridization to the MLS_B probe. Data points and error bars represent mean values and half ranges of duplicate wells.

treating swine waste but attributed their results to the enrichment with resistant bacteria present in the reactor feed. Occurrence of macrolide resistant organisms in swine waste has been reported previously (Chen et al., 2010; Holzel et al., 2010; Jindal et al., 2006). These results suggest that the development of MLS_B resistance can be influenced by historical exposure of the seed sludge to antimicrobials and antimicrobial resistant microorganisms and the presence of antimicrobial resistant microorganisms in the influent. The inoculum used in the reactor of the current study had no known exposure to antimicrobials, and the reactor received sterile synthetic wastewater, so these experiments required the development of tylosin resistance rather than monitoring the accumulation of antimicrobial resistance. It is encouraging that resistance did not develop, as a single base pair change can result in MLS_B resistance in many organisms. Our data do not exclude the possibility that alternative mechanisms of resistance developed, but any alternative mechanisms that were present were not sufficient to maintain or restore performance.

Modeling

The overall microbial population dynamics observed in the ASBR were adequately described by the model (Fig. 2). The modeled abundance of glucose fermenting bacteria was consistently higher than the measured levels of Actinobacteria, which is likely due to the presence of fermentative bacteria belonging to other phyla, including Firmicutes and Chloriflexi. Aceticlastic methanogens, hydrogen-consuming methanogens, and propionate-oxidizing bacteria levels of up to 30%, 10%, and 2%, respectively, were also observed in a glucose-fed upflow anaerobic sludge blanket reactor (Zheng et al., 2006). The 16S rRNA-based quantification used by Zheng et al. (2006) may have underestimated the actual levels of propionate-oxidizing bacteria because the available 16S rRNA targeted probes for propionate-oxidizing bacteria may not target all species within this functional group.

Conclusions

The effects of tylosin vary among different microbial groups of the anaerobic food web. Gram-positive glucose fermenting bacteria (family Propionibacteriaceae) maintained activity in the presence of tylosin concentrations of 167 mg/L. Gram-negative propionate-oxidizing syntrophic bacteria (family Syntrophobacteraceae) and Gram-positive bacteria (genus *Clostridium*), on the other hand, were detected less frequently after tylosin was introduced. This combination of tylosin resistance in glucose fermenting bacteria and inhibition of propionate or butyrate oxidizing syntrophic bacteria resulted in the accumulation of organic acids in an ASBR. The microbial analysis of a glucose-fed ASBR exposed to different concentrations of tylosin showed that the addition of tylosin without the input of

antimicrobial resistance genes did not result in an increase in the prevalence of MLS_B resistance in the reactor.

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