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Growth Kinetics of *Hyphomicrobium* and *Thiobacillus* spp. in Mixed Cultures Degrading Dimethyl Sulfide and Methanol[▽]

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The growth kinetics of *Hyphomicrobium* spp. and *Thiobacillus* spp. on dimethyl sulfide (DMS) and methanol (in the case of *Hyphomicrobium* spp.) in an enrichment culture created from a biofilter cotreating DMS and methanol were studied. Specific growth rates of 0.099 h^{-1} and 0.11 h^{-1} were determined for *Hyphomicrobium* spp. and *Thiobacillus* spp., respectively, growing on DMS at pH 7. These specific growth rates are double the highest maximum specific growth rate for bacterial growth on DMS reported to date in the literature. When the pH of the medium was decreased from pH 7 to pH 5, the specific growth rate of *Hyphomicrobium* spp. decreased by 85%, with a near 100-fold decline in the yield of *Hyphomicrobium* 16S rRNA gene copies in the mixed culture. Through the same pH shift, the specific growth rate and 16S rRNA gene yield of *Thiobacillus* spp. remained similar. When methanol was used as a substrate, the specific growth rate of *Hyphomicrobium* spp. declined much less over the same pH range (up to 30%) while the yield of 16S rRNA gene copies declined by only 50%. Switching from an NH_4^+-N-based source to a NO_3^--N-based source resulted in the same trends for the specific growth rate of these microorganisms with respect to pH. This suggests that pH has far more impact on the growth kinetics of these microorganisms than the nitrogen source. The results of these mixed-culture batch experiments indicate that the increased DMS removal rates observed in previous studies of biofilters cotreating DMS and methanol are due to the proliferation of DMS-degrading *Hyphomicrobium* spp. on methanol at pH levels not conducive to high growth rates on DMS alone.

Dimethyl sulfide (DMS) is a reduced sulfur compound that is emitted from both natural and anthropogenic sources. Natural DMS emissions are largely the result of the cleavage of dimethylsulfoniopropionate (9), the breakdown of the sulfur-containing amino acids methionine and cysteine (9, 11), and the degradation of methoxylated aromatic compounds (3, 9). Anthropogenic DMS emissions tend to be the result of high-temperature industrial processes and are problematic due to the foul smell of DMS and its low odor threshold (34). Industries that are sources of anthropogenic DMS emissions include wastewater treatment (14), aerobic composting (40), animal rendering (23), and kraft pulping (35).

In the environment, microbial degradation can be a significant sink for DMS. In seawater, approximately 90% of the DMS produced is removed biologically before it reaches the atmosphere (21). Removal of DMS in the environment can be carried out by a variety of pathways. Aerobic bacteria, such as *Hyphomicrobium* spp. (7, 27, 36, 45), *Thiobacillus* spp. (6, 19, 42), or *Methylophaga* spp. (10), convert DMS to oxidized inorganic sulfur products such as sulfate and thiosulfate. There are also a wide variety of microorganisms, such as *Pseudomonas* spp. (46), capable of oxidizing DMS to dimethyl sulfoxide (DMSO) (11, 18). Finally, DMS can be eliminated through several anaerobic pathways, with a variety of methanogens (13, 22), sulfate-reducing bacteria (39), phototrophic bacteria (41, 44), and denitrifiers (42) capable of growth on DMS being reported in the literature.

The prevalence of bacteria in the environment capable of growth on DMS has created interest in developing low-cost biotechnological methods to remove DMS from industrial waste gas streams. One possible technology is biofiltration which involves passing waste air through a packed bed of microorganisms. Removal of DMS in these systems, however, has proved to be difficult. This is believed to be due to the acidification of the biofilter bed brought about by the conversion of DMS to sulfate (32).

Previous research by our group investigated the effect of the presence of methanol on DMS removal rates in inorganic biofilters treating DMS inoculated with sludge since industrial biofilters often treat mixtures of waste gases with biofilms composed of mixed microbial communities. It was demonstrated that the DMS degradation rate in biofilters where the pH of the biofilter bed was allowed to acidify naturally over time to pH 5 before the pH was neutralized back to pH 7 could be increased by up to 11-fold with methanol cotreatment (47). The increase in the DMS removal rates was shown to be the result of an order of magnitude increase in the concentration of *Hyphomicrobium* spp., which were also capable of growth on methanol (16). Methanol addition also resulted in a decrease in the full conversion of DMS to sulfate (increase in S^0) and nitrification in biofilters cotreating DMS and methanol compared to the biofilter treating DMS alone, resulting in a decrease in the rate of acidification in these biofilters (48). Finally, it was shown that the DMS removal rate in these

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TABLE 1. Quantitative PCR primers and probes used in this study to quantify the 16S rRNA genes of *Bacteria*, *Hyphomicrobium* spp., *Thiobacillus* spp., and *Chitinophaga* spp.

Microbial group	Oligonucleotide name	Oligonucleotide sequence	Reference or source
Bacteria	BAC Forward	5′-TCCTACGGGAGGCAGCAGT-3′	25
	BAC Probe	5′-FAM-CGTATTACCGCGGCTGCTGGCAC-TAMRA-3′	25
	BAC Reverse	5′-GGACTACCAGGGTATCTAATCCTGTT-3′	25
Hyphomicrobium	Hyp Forward	5′-GGCTCAACCTCGGAACT-3′	16
	Hyp Reverse	5′-CGAATTTCACCTCTACACTAGGAT-3′	16
	Hyp Probe 1	5′-FAM-TGAGTCCGATAGAGGTGGGTGG-TAMRA-3′	16
	Hyp Probe 2	5′-FAM-AGTCTTGAGTCCGGAAGAGG-TAMRA-3′	16
Thiobacillus	Thio Forward	5′-CCTCACGTTATTCGAGCGG-3′	16
	Thio Reverse	5′-ACGCACTCTAGACTGCCA-3′	16
Chitinophaga	Chi Forward	5′-TTRAAGATGGSYGTGCRYC-3′	16
	Chi Reverse	5′-CGCTACATGACATATTCCGCT-3′	16

biofilters could be optimized by adopting step-feeding (49) and pulse-feeding (50) strategies.

This paper focuses on the mechanism behind increased DMS removal rates in biofilters cotreating DMS and methanol. The behavior of an enrichment culture created from a biofilter cotreating DMS and methanol under different conditions relevant to biofilter operations as reported previously was investigated. Batch studies coupled with quantitative PCR (qPCR) methods were conducted to determine the effect of pH and nitrogen source on the kinetics of *Hyphomicrobium* spp., *Thiobacillus* spp., and a group of bacteria closely related to *Chitinophaga* spp. present in the enrichment culture grown on DMS and methanol.

MATERIALS AND METHODS

Establishment of original enrichment culture and subcultures. An enrichment culture was established by inoculating 40 ml of mineral medium (27) in 250-ml glass bottles sealed with Mininert valves (Supelco, Bellefonte, PA) with biomass obtained from a biofilter cotreating DMS and methanol. The enrichment culture was maintained by serial 1:2,500 dilutions of fully grown cultures with fresh medium and by refeeding liquid DMS (0.3 mM initial DMS concentration). This enrichment culture has been maintained for >2.5 years growing in medium at ambient temperature (22 to 24°C), pH 7, and shaken at 150 rpm.

Subcultures of the original enrichment were established for testing the behavior of the culture under different conditions. To test the effect of nitrogen, the 0.4 g/liter NH_4Cl in the mineral medium was replaced with 0.9 g/liter KNO_3. To test different pH conditions, the initial pH of the medium was lowered by adding the necessary volume of a 0.2 M H_2SO_4 solution. When methanol was used as a substrate, polypropylene bottles were substituted for glass bottles, and an initial concentration of 60 mM methanol was used. At least two growth cycles were carried out on subcultures before batch kinetic assays were performed.

Construction of genus- and family-wide phylogenies of *Hyphomicrobium*, *Thiobacillus*, and *Chitinophaga*. 16S rRNA gene sequences clustering within the *Hyphomicrobium* and *Thiobacillus* genera and the *Chitinophaga* family in a previously constructed *Bacteria*-wide 16S rRNA gene clone library of the enrichment culture created from a biofilter cotreating DMS and methanol (16) were reanalyzed. Sequences were aligned using Greengenes (8), and minimum evolution phylogenies were constructed using MEGA 4 with the Kimura two-parameter nucleotide model (38).

Time course batch experiments. Time course batch experiments were carried out in 250-ml glass bottles, sealed with Mininert valves, containing 40 ml of mineral medium at ambient temperature (22 to 24°C) and shaken at 150 rpm. The gas phase concentrations of DMS and methanol were measured using gas chromatography (GC), sulfate formation was measured by ion chromatography, and the quantity of 16S rRNA genes from *Bacteria*, *Hyphomicrobium* spp., *Thiobacillus* spp., and *Chitinophaga* spp. was measured using qPCR. Time course batch experiments for the DMS experiments were performed in triplicate and repeated ($n = 6$ for each condition) while time course batch experiments for methanol were carried out in triplicate once ($n = 3$ for each condition). Biomass samples were taken at approximately 24-h intervals over the course of the approximately 90 h it took for the substrate (DMS or methanol) to be degraded. To determine specific growth rates, the natural logarithm of the number of 16S rRNA gene copies per ml of medium was plotted versus time. Biomass yield estimates were made by converting quantitative PCR yields using estimates of average cellular biovolume, average carbon content of cellular dry mass, and the number of 16S rRNA genes per cell. This approach was taken since the volumes and biomass content available in these studies were relatively small and since pure cultures were not obtained from the enrichment culture.

Analytical methods. Gas phase concentrations of DMS and methanol in the headspace of the batch culture bottles were measured with a gas chromatograph (Varian 3400 Cx; Palo Alto, CA) equipped with a pulse flame photometric detector (PFPD) and flame ionization detector (FID). A DB-1 capillary column was used (internal diameter, 5.0 μm; length, 30 m; film thickness, 0.32 mm). A constant column temperature of 120°C was used with the injection port at 120°C and the detection temperature at 250°C. A 50-μl or 250-μl sample was injected into the GC using a 250-μl gas-tight syringe. Aqueous sulfate ion concentrations were measured by ion chromatography using a Dionex (Sunnyvale, CA) 300 series ion chromatograph equipped with an Ionpac AS11 40-mm column. The eluent flow rate was 1.0 ml/min with the following concentration gradient: 5 mM NaOH for 5 min, increased linearly to 25 mM NaOH over the next 10 min, maintained at 25 mM NaOH for the next 3 min, reduced linearly to 5 mM NaOH over next 2 min, and maintained at 5 mM NaOH over next 5 min.

Quantification of 16S rRNA genes from *Bacteria*, *Hyphomicrobium* spp., *Thiobacillus* spp., and *Chitinophaga* spp. was performed using qPCR. Biomass samples were harvested from the batch reaction mixtures by centrifuging 1.5 ml of the culture at 10,000 × *g* for 5 min and removing the supernatant. Genomic DNA extractions were performed using an Ultraclean Soil DNA Kit (Mo Bio Laboratories, Carlsbad, CA) with the normal extraction procedure. All primers and probes used in this study are listed in Table 1. All qPCRs were carried out using a Lightcycler 2.0 (Roche Diagnostics Corp., Indianapolis, IN). For quantification of 16S rRNA genes from *Bacteria* and *Hyphomicrobium* spp., *Taq* nuclease assays were carried out in a total volume of 20 μl using a Lightcycler TaqMan Master kit (Roche Diagnostics Corp., Indianapolis, IN) with primer concentrations of 0.5 μM and a hydrolysis probe concentration of 0.1 μM. The thermal cycling program consisted of an initial 10-min denaturation at 95°C, followed by 45 cycles of 95°C for 10 s, 55°C for 20 s, and 72°C for 20 s. For quantification of *Thiobacillus* spp. and *Chitinophaga* spp., SYBR green I assays were carried out in a total volume of 20 μl using a Lightcycler FastStart DNA Master Plus SYBR green I kit (Roche Diagnostics Corp., Indianapolis, IN) with primer concentrations of 0.5 μM. For the *Thiobacillus* assay, the cycling program included a preincubation of 10 min at 95°C and 45 cycles of 95°C for 10 s, 52°C for 20 s, and 72°C for 20 s. For the *Chitinophaga* assay, the cycling program included a preincubation of 10 min at 95°C and 45 cycles of 95°C for 10 s, 57°C for 20 s, and 72°C for 20 s. Product purity was verified by melting curve analysis.

RESULTS

Characterization of an enrichment culture from a biofilter and growth on DMS. An enrichment culture was created by

inoculating mineral medium with biomass collected from a biofilter cotreating DMS and methanol and by feeding the culture DMS as the sole organic carbon source. A *Bacteria*-wide Bayesian phylogeny revealed that the enrichment culture was composed of a wide variety of bacteria, and a comparison of this clone library with one constructed from a biofilter treating DMS alone revealed three groups of bacteria (*Hyphomicrobium*, *Thiobacillus*, and *Chitinophaga*) that appear to be tightly correlated to DMS degradation in these systems (16). Minimum evolution phylogenies of the *Hyphomicrobium* and *Thiobacillus* genera and the *Chitinophaga* family reveal that while there is genetic diversity in the 16S rRNA gene sequences within each of these groups, the identified clones from *Thiobacillus* spp. tend to be much less genetically diverse in terms of evolutionary distance than clones from *Hyphomicrobium* spp. and *Chitinophaga* spp. (Fig. 1). Furthermore, a more complete phylogeny constructed from 16S rRNA genes of members of the *Chitinophagaceae* family revealed that the clones identified in our previous work as *Chitinophaga* are closely related to members of this family but form their own distinct group.

The growth of *Hyphomicrobium* spp., *Thiobacillus* spp., and *Chitinophaga* spp. in the enrichment culture with DMS as the sole carbon source was studied. As shown in Fig. 2, the initial DMS degradation rate was low at the beginning but increased greatly throughout the experiment as microorganisms proliferated exponentially with a concomitant decrease in DMS. This was the case for all time course batch experiments performed in this study. In order to determine the specific growth rate (μ), an exponential relationship for biomass growth with time was assumed. Therefore, a plot of the natural logarithm of 16S rRNA gene copy number versus time should yield a straight line where the slope is equal to the specific growth rate. As shown in Fig. 3, this was indeed the case for the example shown, where regression plots of the natural logarithm of 16S rRNA gene copies versus time of *Hyphomicrobium* spp., *Thiobacillus* spp., and *Chitinophaga* spp. all had R^2 values above 0.93. This is representative of all of the time course batch experiments carried out in this study. It is also important to note that, for *Chitinophaga* spp., there was no significant difference in the yield of 16S rRNA gene copy numbers for *Chitinophaga* spp. in negative-control cultures (no DMS) that were run under the same conditions as the DMS-grown mixed cultures. Neither *Hyphomicrobium* spp. nor *Thiobacillus* spp. grew in the negative-control cultures.

Growth kinetics of *Hyphomicrobium*, *Thiobacillus*, and *Chitinophaga* spp. on DMS in enrichment culture. The specific growth rate of *Hyphomicrobium* spp. on DMS was strongly affected by the pH of the mineral medium while the nitrogen source had no significant effect on the specific growth rate of *Hyphomicrobium* spp. (Table 2). Maximum specific growth rate values for *Hyphomicrobium* spp. were achieved at pH 7 in both the NH_4^+-N and NO_3^--N media (0.099 h^{-1} and 0.089 h^{-1}, respectively). At pH 6, for both media there was a significant decrease in the specific growth rates to 0.033 h^{-1} ($P = 0.0015$ and $P = 0.0063$, respectively) and, at pH 5, the specific growth rates were 0.015 h^{-1} and 0.020 h^{-1}, respectively. The same trend was observed for the yield of *Hyphomicrobium* spp., where large declines were observed in the yield with decreasing pH. At pH 7, *Hyphomicrobium* spp. had respective yields in the NH_4^+-N and NO_3^--N media of 1.8×10^7 and 4.0×10^6 16S rRNA gene copies per μmol of DMS consumed, with the NO_3^--N yield being significantly lower than the NH_4^+-N yield ($P = 0.00018$). At pH 6, the yields of *Hyphomicrobium* spp. in the NH_4^+-N and NO_3^--N media were 1.0×10^6 and 1.8×10^5 16S rRNA gene copies per μmol of DMS consumed, respectively, and at pH 5 the respective yields were 1.5×10^5 and 1.6×10^5 16S rRNA gene copies per μmol of DMS consumed. There was no significant difference at the 95% confidence level between any of the *Hyphomicrobium* yields at pH 6 or pH 5, and all four yield values were significantly lower than the yields obtained at pH 7 in the respective media.

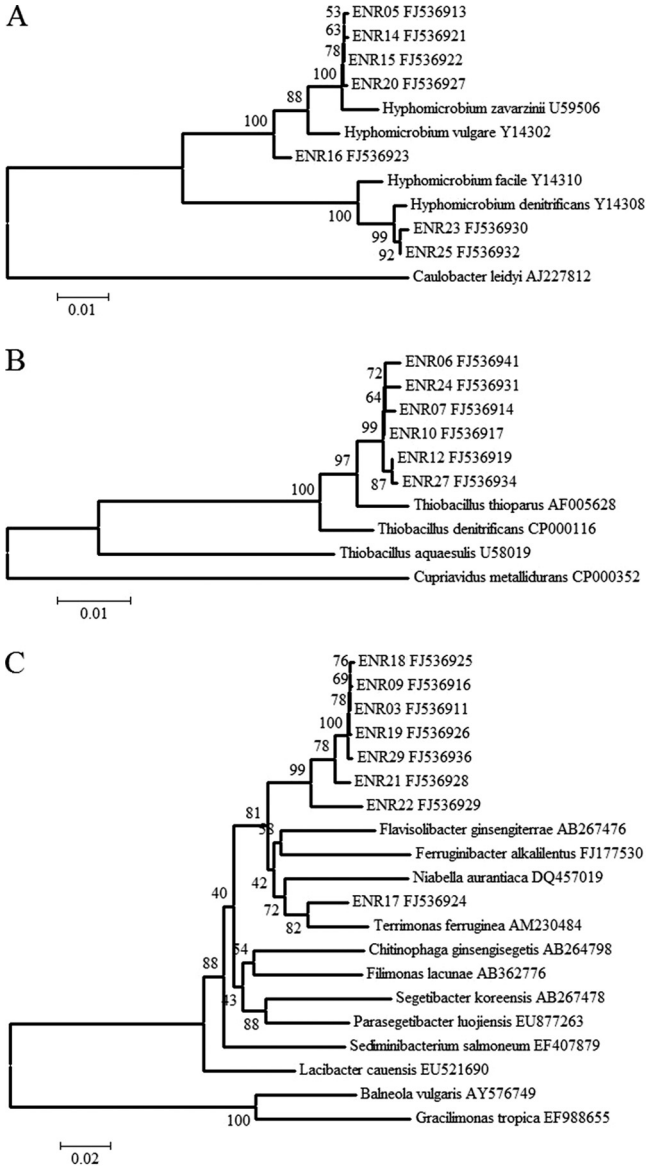

FIG. 1. Genus-wide (A, *Hyphomicrobium*; B, *Thiobacillus*) and family-wide (C, *Chitinophagaceae*) minimum evolution phylogenies of clones identified in an enrichment culture created from a biofilter cotreating DMS and methanol. Clones are denoted ENR and GenBank accession numbers of all sequences used are listed. The scale is in number of substitutions per nucleotide.

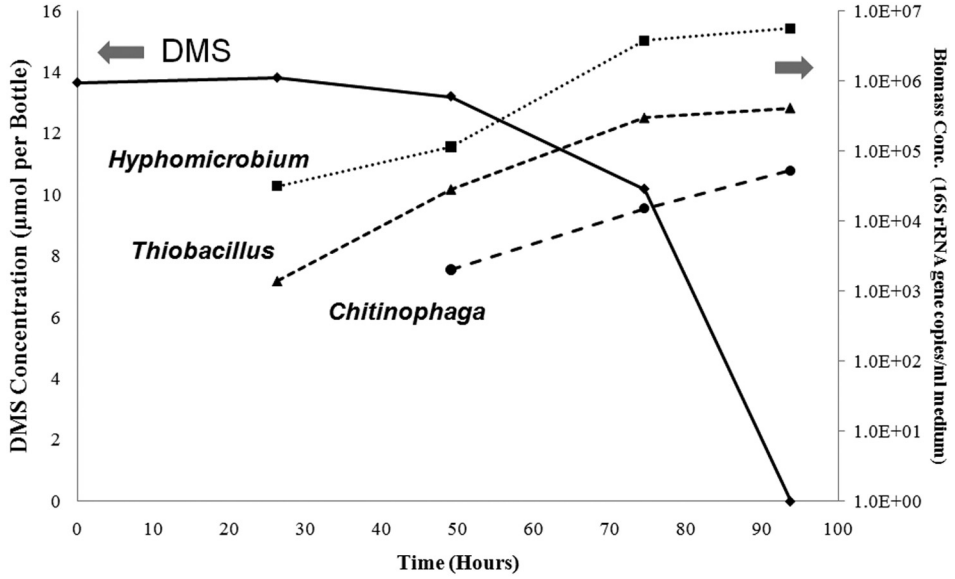

FIG. 2. DMS consumption and growth of *Hyphomicrobium* spp., *Thiobacillus* spp., and *Chitinophaga* spp. in the enrichment culture in an NH_4Cl-based medium at pH 7 versus time.

The specific growth rate of *Thiobacillus* spp. was fairly consistent across the pH range tested. At pH 7, the specific growth rates in the NH_4^+-N and NO_3^--N media were 0.076 h^{-1} and 0.110 h^{-1}, respectively. At pH 6, the specific growth rate in the NH_4^+-N medium was 0.080 h^{-1}, which was not significantly different from the specific growth rate at pH 7 ($P = 0.77$). However, in the NO_3^--N medium the specific growth rate was 0.065 h^{-1}, which was significantly lower than the specific growth rate in the NO_3^--N medium at pH 7 ($P = 0.022$). At pH 5, the specific growth rates in the NH_4^+-N and NO_3^--N media were 0.084 h^{-1} and 0.110 h^{-1}, respectively. Neither of the specific growth rates was significantly different at the 95% confidence level from the values in the respective medium at either pH 6 or pH 7. As for the effect of the nitrogen source on the specific growth rate of *Thiobacillus* spp., at pH 7, the specific growth rate of *Thiobacillus* spp. was significantly higher in the NO_3^--N medium ($P = 0.020$), but at pH 5 and pH 6 there was no significant difference at the 95% confidence level. The yield of *Thiobacillus* spp. was also consistent across the pH ranges tested. The minimum yield of 1.4×10^6 16S rRNA gene copies per µmol of DMS consumed occurred at pH 6 in the NH_4^+-N medium while the maximum yield of 3.5×10^6 16S

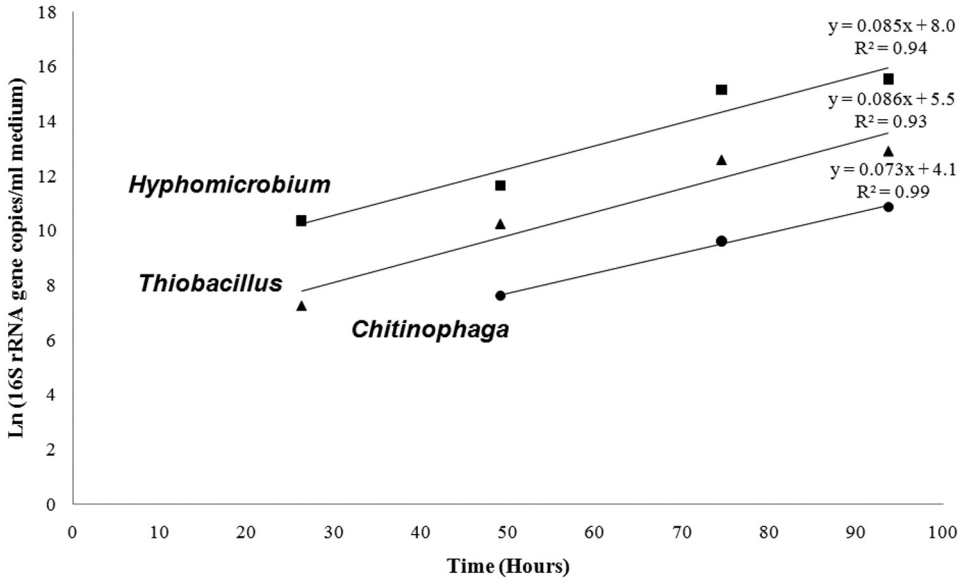

FIG. 3. Plot of the natural logarithm of the number of 16S rRNA gene copies of *Hyphomicrobium* spp., *Thiobacillus* spp., and *Chitinophaga* spp. in the enrichment culture versus time for the same experiment as shown in Fig. 2.

TABLE 2. Effect of pH and nitrogen source on the specific growth rate and yield of *Hyphomicrobium* spp., *Thiobacillus* spp., and *Chitinophaga* spp. in the enrichment culture grown on DMS

Microbial group	Nitrogen source	Specific growth rate (h^{-1}) at the indicated pH[a]			Yield (no. of 16S rRNA gene copies/μmol of DMS consumed) at the indicated pH[a]		
		5	6	7	5	6	7
Hyphomicrobium	NH_4Cl	0.015 (0.015)	0.033 (0.029)	0.099 (0.016)	$1.5\ (1.4) \times 10^5$	$1.0\ (0.9) \times 10^6$	$1.8\ (0.8) \times 10^7$
	KNO_3	0.020 (0.019)	0.033 (0.018)	0.089 (0.022)	$1.6\ (1.1) \times 10^5$	$1.8\ (1.1) \times 10^5$	$4.0\ (1.7) \times 10^6$
Thiobacillus	NH_4Cl	0.084 (0.052)	0.080 (0.033)	0.076 (0.019)	$3.0\ (1.8) \times 10^6$	$1.4\ (0.9) \times 10^6$	$3.5\ (2.4) \times 10^6$
	KNO_3	0.110 (0.04)	0.065 (0.030)	0.110 (0.026)	$3.0\ (2.5) \times 10^6$	$2.2\ (1.9) \times 10^6$	$2.5\ (1.5) \times 10^6$
Chitinophaga	NH_4Cl	0.052 (0.038)	0.044 (0.036)	0.061 (0.011)	$3.3\ (2.5) \times 10^5$	$3.9\ (1.2) \times 10^4$	$1.4\ (0.9) \times 10^6$
	KNO_3	0.039 (0.030)	0.063 (0.021)	0.075 (0.020)	$3.2\ (2.1) \times 10^5$	$2.1\ (1.6) \times 10^5$	$1.5\ (1.1) \times 10^6$

[a] Numbers in parentheses indicate standard deviations.

rRNA gene copies per μmol of DMS consumed occurred at pH 7 in the NH_4^+-N medium. None of the yields of *Thiobacillus* spp. under any of the conditions was significantly different from any of the other yields at the 95% confidence level.

Similarly to *Thiobacillus* spp., the specific growth rates and yields of *Chitinophaga* spp. were also fairly consistent across the pH range tested. At pH 7, the specific growth rates in the NH_4^+-N and NO_3^--N media were 0.061 h^{-1} and 0.076 h^{-1}, respectively. At pH 6, the specific growth rates in the NH_4^+-N and NO_3^--N media were 0.044 h^{-1} and 0.063 h^{-1}, respectively. At pH 5, the specific growth rates in the NH_4^+-N and NO_3^--N media were 0.052 h^{-1} and 0.039 h^{-1}, respectively. There were no significant differences at the 95% confidence level between specific growth values at different pH values or with different nitrogen sources. At pH 7, the yields of *Chitinophaga* spp. in the NH_4^+-N and NO_3^--N media were 1.4×10^6 and 1.5×10^6 16S rRNA gene copies per μmol of DMS consumed, respectively. At pH 6, the yields declined significantly to 3.9×10^4 ($P = 0.0096$) and 2.1×10^5 ($P = 0.0046$) 16S rRNA gene copies per μmol of DMS consumed, respectively, and, at pH 5, the yields were 3.3×10^5 and 3.2×10^5 16S rRNA gene copies per μmol of DMS consumed, respectively. The NH_4^+-N medium showed a statistically significant increase at pH 5 compared to the yield at pH 6 ($P = 0.030$) while there was no significant difference in the yield of *Chitinophaga* spp. in the NO_3^--N medium for these two pH values. Also, both pH 5 yields were significantly lower than the yield in the respective medium at pH 7 ($P = 0.018$ and $P = 0.0079$, respectively). The only significant difference that nitrogen source had on yields of *Chitinophaga* spp. was a higher yield of *Chitinophaga* spp. in the NO_3^--N medium than in the NH_4^+-N medium at pH 6 ($P = 0.037$).

The yields of *Hyphomicrobium* spp. and *Thiobacillus* spp. on DMS in this enrichment culture were compared to the reported yields of *Hyphomicrobium* VS and *Thiobacillus thioparus* Tk-m from the literature as well as the theoretical biomass yield at pH 7 with an NH_4^+-N source calculated from bioenergetics (Table 3). Theoretical yields can be estimated from the free energy released from the oxidation of DMS. This calculation resulted in an estimate of the maximum yield equal to 0.565 electron equivalents (eeq) of cells/eeq of DMS, which corresponds to 1.03 g of cells/g of DMS, assuming 113 g of cells/20 eeq of cells and 20 eeq/mol of DMS (29). Quantitative PCR yields were converted using estimates of the average biovolume of a cell, average carbon content of cellular dry mass, and the number of 16S rRNA genes per cell. For *Hyphomicrobium* and *Thiobacillus*, the average biovolume estimates used were 8.8×10^{-13} cm^3/cell (17) and 8.6×10^{-14} cm^3/cell (20), respectively. To convert biovolume to biomass (carbon content), a conversion factor of 0.22 g of C/cm^{-3} was used (5). No information was found on the amount of 16S rRNA genes per *Hyphomicrobium* cell, so an assumption of 1 16S rRNA gene copy per cell was assumed. For *Thiobacillus*, no information was found on the number of 16S rRNA gene copies per cell for *Thiobacillus thioparus*. However, *Thiobacillus denitrificans* ATCC 25259 was reported to have two 16S rRNA gene copies per cell (4), so this value was assumed as an estimate in our calculations for *Thiobacillus*.

In addition to tracking the degradation of DMS by gas chromatography and the growth of *Hyphomicrobium* spp., *Thiobacillus* spp., and *Chitinophaga* spp. by qPCR, total bacterial 16S rRNA genes were quantified at the end of the time course batch experiments to verify that *Hyphomicrobium* spp. and *Thiobacillus* spp. did account for the bulk of total bacteria and that other bacteria were not responsible for the bulk of the DMS degradation. Furthermore, sulfate analysis was carried out on the medium to determine the extent of the conversion of DMS to sulfate. At pH 7, the ratio of the sum of 16S rRNA gene copies from *Hyphomicrobium* spp. and *Thiobacillus* spp. to total bacterial 16S rRNA genes averaged 1.76 ± 0.73. At pH 6 and pH 5, the average ratio was 1.08 ± 0.94 and 0.97 ± 0.49, respectively. For sulfate analysis, at pH 7 it was determined

TABLE 3. Comparisons of yields of *Hyphomicrobium* and *Thiobacillus* grown on DMS at pH 7 in NH_4^+-N medium

Organism	Yield on DMS		Reference or source
	No. of 16S rRNA gene copies/μmol	Biomass (g of dry wt/g of substrate)[a]	
Hyphomicrobium	1.80E07	0.1[b]	This study
Thiobacillus	3.50E06	0.01[b]	This study
Hyphomicrobium VS		0.26	27
Hyphomicrobium EG		0.31	36
T. thioparus TK-m		0.49	19

[a] The theoretical yield is 1.03. See text for bioenergetics assumptions and evaluations.

[b] Calculated from qPCR data using assumptions (see text).

TABLE 4. Effect of pH and nitrogen source on the specific growth rate and yield of *Hyphomicrobium* spp. in the enrichment culture grown on methanol

pH	Specific growth rate (h^{-1}) with[a]:		Yield (no. of 16S rRNA gene copies/μmol of MeOH consumed) with[a]:	
	NH_4Cl	KNO_3	NH_4Cl	KNO_3
5	0.095 (0.005)	0.111 (0.009)	$4.2\ (0.6) \times 10^6$	$6.2\ (1.5) \times 10^6$
6	0.134 (0.001)	0.148 (0.002)	$6.5\ (3.8) \times 10^6$	$6.3\ (1.6) \times 10^6$
7	0.120 (0.011)	0.136 (0.015)	$1.5\ (0.5) \times 10^7$	$1.2\ (0.1) \times 10^7$

[a] Numbers in parentheses indicate standard deviations.

TABLE 5. Comparison of yields of *Hyphomicrobium* grown on methanol at pH 7 in NH_4^+-N media

Organism	Yield on methanol		Reference or source
	No. of 16S rRNA gene copies/μmol	Biomass (g of dry wt/g of substrate)[a]	
Hyphomicrobium	1.2E07	0.13[b]	This study
Hyphomicrobium VS		0.38–0.45	27

[a] The theoretical yield is 0.74. See text for bioenergetics assumptions and evaluations.
[b] Calculated from qPCR data using assumptions (see text).

that the increase in sulfate in the batch reactors meant that the conversion of DMS to sulfate was 107% ± 1%. Determination of the percent conversion of DMS to sulfate was not feasible for the pH 6 and pH 5 experiments due to the use of H_2SO_4 to lower the pH of the mineral medium, which resulted in high background sulfate levels.

Growth kinetics of *Hyphomicrobium* spp. on methanol in enrichment culture. The growth of *Hyphomicrobium* spp. in the enrichment culture was further tested with methanol as a substrate under the same conditions used for growth with DMS (Table 4). The pH of the mineral medium had only a slight effect on the specific growth rate of *Hyphomicrobium* spp. for the range tested. At pH 7, the specific growth rates of *Hyphomicrobium* spp. in the NH_4^+-N and NO_3^--N media were 0.12 h^{-1} and 0.14 h^{-1}, respectively. At pH 6, the specific growth rates of *Hyphomicrobium* spp. in the NH_4^+-N and NO_3^--N media were 0.13 h^{-1} and 0.15 h^{-1}, respectively, but neither growth rate was significantly different at the 95% confidence level from the specific growth rate in the respective medium at pH 7. At pH 5, the specific growth rates for the NH_4^+-N and NO_3^--N media decreased to 0.095 h^{-1} and 0.11 h^{-1}, respectively. Both were a significant decrease from the respective value at pH 6 ($P = 0.018$ and $P = 0.0027$, respectively). There were no significant differences at the 95% confidence level in the specific growth rate as a result of nitrogen source. As for yield, at pH 7, the yields of *Hyphomicrobium* spp. in the NH_4^+-N and NO_3^--N media were 1.5×10^7 and 1.2×10^7 16S rRNA gene copies per μmol of methanol consumed, respectively. At pH 6, the yields of *Hyphomicrobium* spp. in the NH_4^+-N and NO_3^--N media were estimated to be 6.5×10^6 and 6.3×10^6 16S rRNA gene copies per μmol of methanol consumed, respectively. This represented a significant decrease for the NO_3^--N medium ($P = 0.0034$) while the difference was not significant for the NH_4^+-N medium. At pH 5, the yields of *Hyphomicrobium* spp. in the NH_4^+-N and NO_3^--N media were 4.2×10^6 and 6.2×10^6 16S rRNA gene copies per μmol of methanol consumed, respectively. Neither of these was significantly different at the 95% confidence level from the respective yields obtained at pH 6, but both were significantly lower than the respective yields at pH 7 ($P = 0.034$ and $P = 0.0030$, respectively). As for the effect of nitrogen source on yield, there were no significant differences at any pH value at the 95% confidence level.

The yield of *Hyphomicrobium* spp. on methanol in this enrichment culture was compared to the reported yield of *Hyphomicrobium* VS from the literature as well as to the theoretical biomass yield at pH 7 with an NH_4^+-N nitrogen source calculated from bioenergetics (Table 5). Theoretical yields were estimated from the free energy released from the oxidation of methanol. This calculation resulted in an estimate of the maximum yield equal to 0.699 eeq of cells/eeq of methanol, which corresponds to 0.741 g of cells/g of methanol, assuming 113 g of cells/20 eeq of cells and 6 eeq/mol of methanol (29). Quantitative PCR yields were converted using estimates of the average biovolume of a cell, average carbon content of cellular dry mass, and the number of 16S rRNA genes per cell. For *Hyphomicrobium*, the average biovolume was estimated to be 8.8×10^{-13} cm^3/cell (17). To convert biovolume to biomass (carbon content) a conversion factor of 0.22 g of C/cm^{-3} was used (5). No information was found on the number of 16S rRNA genes per *Hyphomicrobium* cell, so an assumption of 1 16S rRNA gene copy per cell was assumed.

As with the enrichment culture grown on DMS, total bacterial 16S rRNA genes were quantified at the end of the experiment to confirm that *Hyphomicrobium* spp. accounted for a significant fraction of the total bacterial 16S rRNA gene copies. At pH 7, the ratio of 16S rRNA gene copies from *Hyphomicrobium* spp. to total bacterial 16S rRNA genes averaged 1.09 ± 0.68. At pH 6 and pH 5, the average ratios were 1.06 ± 0.19 and 0.82 ± 0.27, respectively.

DISCUSSION

The microbial degradation of DMS is an important process in both the natural environment and some engineered biotechnological systems. In this study, it was revealed that, of the three microbial groups previous identified to be tightly linked to DMS degradation in biofilters treating DMS, only *Hyphomicrobium* spp. and *Thiobacillus* spp. were actually growing on DMS while the third group of bacteria, *Chitinophaga* spp., was likely growing on some other substrate. Given that other *Chitinophaga* spp. have been reported to grow on chitin (30) and carboxymethyl cellulose (43), it is possible that these bacteria may be contributing to carbon cycling within the microbial community rather than DMS degradation directly by breaking down complex organic compounds originating from other bacteria.

The maximum specific growth rates for *Hyphomicrobium* spp. (0.099 h^{-1}) and *Thiobacillus* spp. (0.11 h^{-1}) on DMS were double the highest maximum specific growth rates reported in the literature for microorganisms growing on DMS, i.e., 0.05 h^{-1} for *Methylophaga sulfidovorans* (10), 0.05 h^{-1} for *T. thioparus* Tk-m (19), and 0.04 h^{-1} for *Hyphomicrobium* VS (27). This result is not likely explained by the use of qPCR to

estimate biomass (versus volatile suspended solids [VSS], for example) since the maximum specific growth rate measured in this study for *Hyphomicrobium* spp. on methanol (0.15 h^{-1}) is similar to the maximum specific growth rates on methanol for *Hyphomicrobium* VS (0.14 h^{-1}) (27) and *Hyphomicrobium* X (0.10 h^{-1}) (15). The increased specific growth rates of *Hyphomicrobium* spp. and *Thiobacillus* spp. on DMS in this enrichment culture may be the result of the mixed culture improving growth on DMS relative to pure cultures. Mixed cultures provide several situations where microbial growth of a particular organism may be enhanced as a result of synergies among members of the community. The removal of inhibitory compounds or the production of vitamins or other growth factors by other microorganisms (24) may improve growth compared to a pure culture growing in a defined medium. However, there have been relatively few pure-culture kinetic studies carried out with DMS as the substrate, and our observation of higher specific growth rates may reflect this and the utility of qPCR for estimating growth kinetics *in situ*.

Biomass yields of both *Hyphomicrobium* spp. and *Thiobacillus* spp. were significantly lower in the enrichment culture at pH 7 than both the theoretical yield calculated using bioenergetics and biomass yields of pure cultures grown on DMS reported in the literature. This is a product of the enrichment culture being a mixed culture, the experimental method employed, and how the yield was calculated. Since the enrichment culture is a mixed culture, both *Hyphomicrobium* spp. and *Thiobacillus* spp. are growing on DMS, and there is no way to determine how much of the DMS substrate each group is consuming, leading to an underestimation of their respective yields. The experimental method also leads to an underestimation of the yield due to losses that may accrue in the extra processing steps involved compared to VSS measurements, particularly the genomic DNA extraction. Finally, the biomass yield is calculated using estimates of biovolume, cell carbon, and 16S rRNA gene copy number per genome.

A possible concern raised by the low biomass yields is that there is another microorganism degrading DMS in the enrichment culture. There are several results that suggest that this is not the case. First, when methanol was the substrate, a similar, approximately 65% underestimate of the biomass yield was observed at pH 7 for *Hyphomicrobium* spp. compared to the biomass yield of *Hyphomicrobium* VS in pure culture. This suggests that the biomass yield underestimation is mainly an artifact of experimental methodology. Second, DMS was completely converted to sulfate in the pH 7 experiments, and 16S rRNA gene copies from *Hyphomicrobium* spp. and *Thiobacillus* spp. accounted for the bulk of bacterial 16S rRNA genes in the batch experiments. Given that only bacteria are known to carry out the aerobic pathway of converting DMS to sulfate (as opposed to DMSO), this makes the likelihood of another microorganism being responsible for significant DMS removal in the enrichment culture unlikely.

The effect of the pH of the medium had different effects on the kinetics of *Hyphomicrobium* spp. and *Thiobacillus* spp. While there was a clear deterioration in the ability of *Hyphomicrobium* spp. to grow on DMS as the pH decreased from pH 7 to pH 5, *Thiobacillus* spp. maintained their ability to grow on DMS as the pH decreased from pH 7 to pH 5. There is very little information on the effect of pH on the growth kinetics of *Hyphomicrobium* spp. and *Thiobacillus* spp. in the literature. In the case of *Hyphomicrobium* spp., experiments on pure cultures were all carried out at pH 7 only. However, a study of an enrichment culture believed to be dominated by *Hyphomicrobium* spp. (observed by microscopy) had a maximum DMS degradation rate somewhere between pH 6.0 and 7.0. When the pH of the enrichment culture was decreased to 5.0, the DMS degradation rate decreased to 50% of the maximum DMS degradation rate (32). This is consistent with observations in the enrichment culture in this study that showed that the specific growth rate and, by extension, the DMS degradation rate, of *Hyphomicrobium* spp. decreased by up to 85% when the pH was decreased from pH 7 to pH 5. Furthermore, no sulfate inhibition was observed in the *Hyphomicrobium* enrichment culture for sulfate concentrations below 24 g SO_4^{2-}/liter (33), a sulfate concentration that exceeds any sulfate concentration measured in this study by at least an order of magnitude, suggesting that the decline in the specific growth rate is due to pH rather than sulfate inhibition. As for *Thiobacillus* spp., the specific growth rate of *T. thioparus* Tk-m was reported to decrease by 50% as the pH decreased from pH 7.7 to pH 6.1, with no growth occurring at pH 5.6 (19). However, in a biotrickling filter study, where nearly 100% removal efficiencies were achieved and the microbial community was typically composed of more than 10% *Thiobacillus* spp. and less than 0.1% *Hyphomicrobium* spp. on a total bacterial 16S rRNA gene basis, the DMS degradation rate remained robust (~90% removal efficiency) as the pH of the recirculation medium was adjusted to pH 5 over the course of 1 day (31). Given that these experiments were carried out only over the course of a day, it is unknown if the results would have been sustainable in the long term, but the batch data in this study suggest that there are *Thiobacillus* spp. that can grow equally well at pH 5 and pH 7 over the long term.

While decreasing the pH of the medium from pH 7 to pH 5 had a deleterious effect on the growth kinetics of *Hyphomicrobium* spp. on DMS, it had only a minimal effect on the growth kinetics of *Hyphomicrobium* spp. on methanol. It has long been known that *Hyphomicrobium* spp. could be enriched from environmental samples equally quickly in media at pH values between 5.5 and 8.0 (2). This suggests that that the specific growth rates for *Hyphomicrobium* spp. are similar within this pH range, which is consistent with results in this study for *Hyphomicrobium* spp. growing on methanol.

The difference with respect to pH in growth rates on methanol and DMS for *Hyphomicrobium* spp. could be the result of different pH optima of the enzymes involved in the metabolic pathway of these compounds. In *Hyphomicrobium* spp., methanol is first oxidized to formaldehyde by a methanol dehydrogenase (12) while DMS is oxidized to methyl mercaptan and formaldehyde by a DMS monooxygenase (7, 36), and methyl mercaptan is further oxidized to hydrogen sulfide and formaldehyde by a methyl mercaptan oxidase (37). Methanol dehydrogenase is a well-studied enzyme in the literature; methanol oxidation occurs in the periplasmic space via an electron transport chain, with the initial step of methanol dehydrogenase oxidizing cytochrome c_L at a pH optimum of pH 7.0 (1). However, methanol dehydrogenases have been shown to remain stable and retain their activity after incubation for 45 h in buffer from pH 5 to pH 10, with the optimum activity occurring

for samples incubated at pH 6 (26). This and the fact that *Hyphomicrobium* spp. can be isolated almost equally as quickly from environmental samples from pH 5.5 to pH 8.0 suggest a wide pH range in which methanol dehydrogenase is active *in vivo* (2). Enzymatic studies on the metabolism of DMS by *Hyphomicrobium* spp. are limited. De Bont et al. (7) performed the first studies on cell extracts and assayed both DMS monooxygenase and methyl mercaptan oxidase at pH 7.2 but reported low activities for DMS monooxygenase that may have been the result of suboptimal assay conditions. Suylen et al. (37) reported that the pH optimum for methyl mercaptan oxidase in *Hyphomicrobium* EG was pH 8.2. It is possible that the higher pH optimum of methyl mercaptan oxidase and, perhaps of DMS monooxygenase, results in the more rapid decline of the specific growth rate of *Hyphomicrobium* spp. on DMS than on methanol as the pH decreases from pH 7 to pH 5.

The implications of these results on engineered systems are twofold. First, they highlight the importance of pH control in biofiltration and other biotechnological systems designed to remove DMS where the microbial community is comprised of a significant quantity of *Hyphomicrobium* spp. This was evident in our previous study in which the biofilter treating DMS alone was characterized by chronically low DMS removal rates and removal efficiencies in a community where *Hyphomicrobium* spp. accounted for 10% of the bacterial population on a 16S rRNA gene basis (16). Second, these results show that in systems where pH may be difficult to control, such as traditional biofiltration systems, methanol addition can lead to higher DMS removal rates and removal efficiencies under conditions that are not conducive to high growth rates of *Hyphomicrobium* spp. on DMS alone. In our previous biofiltration study, the specific growth rate of the microbial community was experimentally determined to be 0.012 h^{-1} (49). Assuming an air-water partition coefficient of 0.07 for DMS (28), the approximate aqueous concentration of DMS in our batch studies is 300 μM while the approximate concentration of DMS in the biofilm of our biofilters, with an inlet DMS concentration of 20 parts per million by volume (ppmv), would be approximately 10 μM. The half-velocity constant, K_S, of the biofilter microbial community was determined to be 0.2 μM (49), but even if the biofilter microbial community had a K_S on DMS similar to that of *Hyphomicrobium* VS (3 μM) (27), it would be expected that the specific growth rate at pH 7 would be approximately 0.07 h^{-1}, not 0.012 h^{-1}, which is a value that is more analogous to the specific growth rate at pH 5 in the batch studies. In other words, the pH conditions in these biofilters resulted in much lower specific growth rates than can be achieved by *Hyphomicrobium* spp. at pH 7. In the biofilters cotreating DMS and methanol in our previous study, *Hyphomicrobium* spp. were capable of growing at much higher specific growth rates on methanol than on DMS throughout the pH regime that was employed in these biofilters. This resulted in the proliferation of DMS-degrading *Hyphomicrobium* spp. that consumed both DMS and methanol, with the increase in *Hyphomicrobium* spp. being proportional to the increase in DMS degradation in these systems (16). This suggests that these *Hyphomicrobium* spp. still grow at a lower than optimal specific growth rate on DMS in the presence of methanol but that methanol addition results in an increase in the concentration of *Hyphomicrobium* biomass that leads to increased DMS degradation in these biofilters.

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