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Microbial Community Degradation of Widely Used Quaternary Ammonium Disinfectants

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Benzalkonium chlorides (BACs) are disinfectants widely used in a variety of clinical and environmental settings to prevent microbial infections, and they are frequently detected in nontarget environments, such as aquatic and engineered biological systems, even at toxic levels. Therefore, microbial degradation of BACs has important ramifications for alleviating disinfectant toxicity in nontarget environments as well as compromising disinfectant efficacy in target environments. However, how natural microbial communities respond to BAC exposure and what genes underlie BAC biodegradation remain elusive. Our previous metagenomic analysis of a river sediment microbial community revealed that BAC exposure selected for a low-diversity community, dominated by several members of the *Pseudomonas* genus that quickly degraded BACs. To elucidate the genetic determinants of BAC degradation, we conducted time-series metatranscriptomic analysis of this microbial community during a complete feeding cycle with BACs as the sole carbon and energy source under aerobic conditions. Metatranscriptomic profiles revealed a candidate gene for BAC dealkylation, the first step in BAC biodegradation that results in a product 500 times less toxic. Subsequent biochemical assays and isolate characterization verified that the putative amine oxidase gene product was functionally capable of initiating BAC degradation. Our analysis also revealed cooperative interactions among community members to alleviate BAC toxicity, such as the further degradation of BAC dealkylation by-products by organisms not encoding amine oxidase. Collectively, our results advance the understanding of BAC aerobic biodegradation and provide genetic biomarkers to assess the critical first step of this process in nontarget environments.

Benzalkonium chlorides (BACs) are prominent members of quaternary ammonium compounds (QACs), a widely used, broad-spectrum class of disinfectants. As a result of their extensive use in a variety of environmental (e.g., as pesticides in agriculture or hand sanitizers in households) and clinical settings, BACs are frequently detected in natural environments (1) and in the influent of wastewater treatment plants (WWTPs) at concentrations of up to 6 mg/liter (2, 3). BACs are cytoplasmic membrane disruption agents capable of inhibiting cell growth at concentrations as low as 1 mg/liter (4–6). Hence, BACs can be toxic to life when present in nontarget environments, such as WWTPs, freshwater ecosystems, and sediments. In addition, it has been suggested that BACs promote antibiotic resistance in microbial pathogens (7–9). Following their primary application, residual BACs typically accumulate in municipal sewage systems (10, 11); therefore, BAC biodegradation (detoxification) by microorganisms within WWTPs is a desirable process that could reduce potential risks to public and environmental health.

A few studies have identified microorganisms metabolizing BACs and described BAC biodegradation pathways based on biochemical assays. For instance, *Aeromonas hydrophila* and *Bacillus niabensis* can metabolize BACs as a sole carbon and energy source (12, 13). Metabolite analysis suggested that these bacteria transform BACs to benzyldimethylamine (BDMA), a product 500 times less toxic than BACs (14), and a long-chain alkyl group by dealkylation. Although dialyzed cell extract assays have identified amine dehydrogenase and monooxygenase functions involved in cleaving (dealkylating) C_{alkyl}-N bonds (15, 16), the exact gene(s) encoding enzymes for BAC degradation remains unknown (14). Further, although the previous isolate-based studies have offered valuable insights into BAC biotransformation, complex microbial communities, rather than individual organisms, control the fate of BACs in natural and engineered systems. Accordingly, understanding how whole microbial communities adapt to and degrade BACs is important for reliable monitoring and optimization of BAC detoxification processes within WWTPs and natural ecosystems.

The microbial community analyzed in this study originated from a river sediment inoculum that was incubated under aerobic conditions for 3 years with a mixture of BACs as the sole carbon and energy source (14, 17). Previous biochemical analysis of the whole community (14) and individual isolates (17) showed that all BAC constituents are transformed primarily by dealkylation into BDMA and an alkyl chain. A subsequent metagenomic study revealed that the community was highly enriched in members of the *Pseudomonas* genus, most notably *Pseudomonas nitroreducens*, and a versatile repertoire of genes bioinformatically inferred to facilitate biodegradation of BAC or its metabolites, including monooxygenases, dioxygenases, amine dehydrogenases, and amine oxidases (17).

Although the previous metagenomic findings revealed an array

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of organisms and genes potentially involved in community BAC degradation, their actual activities during BAC biodegradation remain elusive. In the present study, we combined the available metagenomic data with time-series metatranscriptomic data collected during a 3.5-day bioreactor feeding cycle to identify candidate genes involved in biodegradation based on their expression profile during BAC exposure, i.e., expression is stimulated by the presence of BAC and repressed when BAC is degraded/disappeared, and reveal microbial interactions controlling the fate of BACs. Predictions based on metatranscriptomic data were validated using functional characterization of isolates and biochemical assays. Therefore, the present study also represents a reference example of how omics techniques and bioinformatics can guide specific functional experiments and identify candidate genes for genetic manipulation.

MATERIALS AND METHODS

Characterization of BAC degradation by the microbial community in the bioreactor. The microbial community analyzed in this study was incubated in a batch-fed bioreactor under aerobic conditions for 3 years (14 days of retention time) with a mixture of BACs as the sole carbon and energy source as described previously (7, 14, 17). Briefly, the inoculum originated from estuarine sediment that was contaminated with metals and numerous organic pollutants (18). The community was developed in an aerobic batch-fed bioreactor in minimal salt medium supplemented with a 60:40 mixture of benzyldimethyldodecylammonium chloride and benzyldimethyltetradecylammonium chloride (C_{12}BDMA-Cl and C_{14}BDMA-Cl, respectively; Sigma-Aldrich) as the sole carbon and energy sources plus NH_4NO_3 as a supplemental nitrogen source (14). To characterize community BAC degradation, triplicate samples were taken from the bioreactor at 0, 0.5, 3, 6, 9, 12, 15, 24, 48, and 72 h during a typical feeding cycle (cycle duration, 84 h). The feeding cycle analyzed in the current study was randomly selected during the multiyear incubation (i.e., bioreactor incubation continued uninterrupted before and after this representative cycle).

Growth of *P. nitroreducens* strain B on BACs. A single colony of *P. nitroreducens* strain B from cells growing on a 1/10-strength tryptic soy agar plate (1/10 TSA) supplemented with 50 mg/liter of BAC mixture was used to inoculate LB medium or 1/10-strength tryptic soy broth (1/10 TSB) with no BACs and was incubated overnight at room temperature with shaking. Cells were centrifuged, the supernatant was removed, and cells subsequently were washed twice with 1× phosphate-buffered saline (PBS) to remove any residual carbon source from growth in LB-TSB media. Washed cells were diluted 1:100 into 8 ml of 1/2-strength Stanier's mineral salts basal (MSB) medium containing 20 mM Na_2HPO_4, 20 mM KH_2PO_4, 425 μM nitrilotriacetic acid, 1.2 mM $MgSO_4$, 225 μM $CaCl_2$, 75 nM $(NH_4)_6Mo_7O_{24}$, 3.5 μM $FeSO_4$, 3.8 mM $(NH_4)_2SO_4$, and a mixture of trace elements (19) and supplemented with 50 mg/liter of BAC mixture (143 μM) as a sole carbon and energy source. Cell growth was measured at 600 nm using a spectrophotometer and a protein assay using a Thermo Scientific Pierce bicinchoninic acid (BCA) protein assay reagent kit.

Analytical methods. C_{12}BDMA-Cl and C_{14}BDMA-Cl concentrations were analyzed by high-performance liquid chromatography (HPLC) with an Agilent Eclipse XDB C_{18} column (4.6 mm by 150 mm; 5 μm). The mobile phase consisted of 40% water with 0.1% trifluoroacetic acid and 60% acetonitrile with 0.05% trifluoroacetic acid at a flow rate of 1 ml/min. The column was kept at 35°C, and UV absorbance was monitored at 254 nm. Retention times of C_{12}BDMA-Cl and C_{14}BDMA-Cl were 3.5 and 6.4 min, respectively. BDMA was quantified in a similar manner, with UV absorbance measured at 210 nm using a Waters Spherisorb C_8 column (4.6 mm by 250 mm; 5 μm) and a retention time of 4.4 min. All samples were mixed with acetonitrile (1:1 by volume) prior to centrifugation and subsequent BAC or BDMA measurement. Spectrophotometric analyses were performed with a Cary 3E UV-visible spectrophotometer. Protein concentration was measured with a Pierce (Rockford, IL) BCA protein assay reagent kit. Soluble chemical oxygen demand (sCOD) was measured as described previously (7) after culture samples were centrifuged for 10 min at 14,000 rpm and filtered using 0.2-μm polytetrafluoroethylene (PTFE) filters.

Microbial community RNA and isolate DNA extraction and sequencing. Mixed-culture suspension (50 ml) was filtered through a 0.22-μm Sterivex filter (Millipore), and total RNA was extracted from the material collected on the filter using an organic extraction method (20). Lysis buffer (50 mM Tris-HCl, 40 mM EDTA, 0.75 M sucrose) was added to the filters with 1 mg/ml lysozyme and subsequently incubated for 30 min at 37°C. A second 2-h incubation at 55°C was performed after the addition of 1% SDS and 10 mg/ml proteinase K. Acid phenol and chloroform extractions were performed twice on the lysates, and RNA was isolated using filter columns from the mirVANA RNA isolation kit (Ambion), washed twice by following the manufacturer's instructions, and eluted in Tris-EDTA buffer. DNase treatment was performed using the TURBO DNA-free kit (Ambion, Austin, TX), followed by rRNA depletion by subtractive hybridization of rRNA (MICROBExpress; Ambion). Enriched mRNA samples were amplified with the MessageAmp II-Bacteria kit (Ambion). The resulting antisense RNA (aRNA) was reverse transcribed with random hexamer primers and the SuperScript II reverse transcriptase kit (Invitrogen) and was purified with the MinElute DNA cleanup kit (Qiagen). Unless otherwise noted, all kits were used by following the manufacturer's instructions. Quality and quantity of nucleic acids during the cDNA preparation protocols were monitored using the Agilent RNA 6000 Pico kit (Agilent), and Qubit RNA assay kit (Invitrogen). The resulting cDNA libraries were sequenced (150-bp single-end reads) using the Illumina GA II sequencer at the Los Alamos National Laboratory Genomics Facility. DNA of pure isolates was extracted as previously described (21) and sequenced on the Ion Torrent platform (Life Technologies) using the 316 chip.

Sequence data analysis. The raw sequencing reads were trimmed using a $Q = 15$ Phred quality score cutoff using SolexaQA (22); sequences with consecutive nucleotides (homopolymers; $n \geq 9$) also were removed from further analysis. All trimmed reads first were searched against 5S (23) and 16S and 23S rRNA gene databases (24) using BLASTn with a bit-score cutoff of 40. The remaining (non-rRNA sequences) reads were searched against all bacterial and archaeal genome sequences available in the NCBI database (ftp://ftp.ncbi.nih.gov/; accessed February 2012; later versions of the NCBI genome database did not alter our results substantially), as well as those determined as part of this study, to assign transcripts to known genera using BLASTn with a cutoff of >80% identity and 50% query length coverage. BLASTn was run with the parameters $X = 150$, $q = -1$, and $F = F$, with the remaining parameters at default settings. To provide a quantitative assessment of the relative contribution of each genus to the community transcriptome, the number of reads matching to each genus was divided by the total number of matches assigned to all genera, which was normalized for the size of different metatranscriptomic data sets.

Protein-coding genes recovered in companion shotgun metagenomic data sets (SRA accession no. SRR639751) were described previously (17). The amino acid sequences of the protein-coding genes were functionally annotated based on the SEED subsystems (25) using BLASTp with a 30% amino acid sequence identity and 50% query length coverage cutoff for a match. Unassembled metatranscriptomic reads (non-rRNA sequences) were mapped on the protein-coding genes with at least 95% identity and 50% query length coverage (BLASTn) to estimate the abundance of protein-coding genes in the metatranscriptomic data sets. The lengths of the matching reads assigned to a gene were summed and divided by the length of the corresponding gene sequence to normalize for the gene length, and the resulting value subsequently was expressed as *X* coverage per 100 Mb of non-rRNA sequences to provide relative gene abundance independent of the size of the metatranscriptomics data set.

DNA reads obtained from the sequenced isolates were trimmed as

TABLE 1 Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Description or sequence	Source
Strains		
P. nitroreducens B	Strain that cleaves BAC to BDMA	This study
E. coli NEB 5α	Host strain for plasmid construction	NEB
E. coli Rosetta 2(DE3)	Host strain for enzyme overexpression	Novagen
Plasmids		
pET21a	Amp^r; overexpression vector	Novagen
pET-KJ911918	Amp^r; pET-21a containing the amine oxidase gene from *P. nitroreducens* strain B	This study
Primers		
AMO-F	PCR amplification of 1,458-bp open reading frame of the amine oxidase gene from *P. nitroreducens* strain B; TTTGAATTCATGGCCGATGAAAAAGACGAC	This study
AMO-R	PCR amplification of 1,458-bp open reading frame of the amine oxidase gene from *P. nitroreducens* strain B; TTTAAGCTTTCATCCCAGCAGC	This study

described above and assembled as previously described (26). Protein-coding genes (longer than 300 bp) were identified using GeneMarkS (v. 4.6b) (27) and functionally annotated using SEED subsystems and BLASTp searches against GenBank, as outlined above.

Cloning and heterologous expression. The primers (Integrated DNA Technologies, Coralville, IA) described in Table 1 were used to amplify target sequences from genomic DNA with the Promega polymerase (Promega, Madison, WI). Sequences were ligated into EcoRI and HindIII sites of the pET-21a vector (Invitrogen Corp., Carlsbad, CA). The resulting recombinant plasmids were transformed into *Escherichia coli* NEB 5α (New England BioLabs, Ipswich, MA) for maintenance or into *E. coli* Rosetta 2(DE3) (Novagen, CA) for overexpression (Table 1). Clones in *E. coli* Rosetta 2(DE3) were overexpressed by growing single colonies in LB medium supplemented with ampicillin (100 mg/liter) and chloramphenicol (30 mg/liter) until an optical density at 600 nm (OD_{600}) of 0.4 to 0.6 at 37°C with shaking. Isopropyl-β-D-thiogalactopyranoside (IPTG) (0.2 mM) was added to induce expression, and cultures were incubated at 16°C with shaking for 15 h. *E. coli* Rosetta 2(DE3) containing pET-21a with no insert was used as the control in overexpression assays. Enzyme activity assays were performed with both whole cells and cell extracts. *P. nitroreducens* strain B was grown on 140 μM BAC with the addition of 10 mM glucose to further stimulate growth and obtain more biomass (compared to growth on BACs as the sole carbon substrate). BAC disappearance was monitored throughout incubation with HPLC. Cells grown on glucose only were used as negative controls.

Enzyme activity assays. Cells were suspended in phosphate buffer (pH 7.4, 20 mM) and passed twice through a French pressure cell (20,000 lb/in^2). The cell lysate was clarified by centrifugation (20,000 × *g*, 4°C, 20 min), and the supernatant was used for crude cell extracts. For dialysis, the supernatant was placed in Slide-A-Lyzer (Pierce) dialysis cassettes and incubated at 4°C overnight in 1 liter of phosphate buffer (pH 7.4, 20 mM). All crude cell extract assays were performed at room temperature in phosphate buffer (pH 7.4, 20 mM). Typical assays contained 0.02 to 0.5 mg protein and either C_{12}BDMA-Cl or C_{14}BDMA-Cl (50 to 100 μM) in a total volume of 1 ml with or without FAD (100 μM) as a cofactor. The reaction was stopped by the addition of equal volumes of acetonitrile containing 1% trifluoroacetic acid, which precipitated the protein. Substrate disappearance was monitored by HPLC to calculate specific activities. All of the mixtures were clarified by centrifugation before HPLC analysis.

Nucleotide sequence accession numbers. The metatranscriptome data used in this study were deposited in GenBank under the accession numbers SRR955467 ($B_{0\ h}$), SRR955468 ($B_{0.5\ h\text{-}1}$), SRR955469 ($B_{0.5\ h\text{-}2}$), SRR955498 ($B_{0.5\ h\text{-}3}$), SRR955499 ($B_{12\ h}$), and SRR955500 ($B_{48\ h}$). The whole-genome sequences of *Pseudomonas nitroreducens* isolates can be found under the accession numbers JNFO00000000 (*P. nitroreducens* DPB) and JNFN00000000 (*P. nitroreducens* B). The locus identifier for the amine oxidase is KJ911918.

RESULTS

Characteristics of BAC degradation by the microbial community. High-performance liquid chromatography (HPLC) was used to determine BAC concentration during a representative bioreactor feeding cycle. While the feeding cycle duration was 84 h, the majority of BACs were removed within 12 h (detection limit, 1 mg/liter) (Fig. 1). Soluble chemical oxygen demand (sCOD) was determined to corroborate the level of BAC degradation. Consistent with BAC disappearance, sCOD decreased from 301 mg/liter at 0.5 h to 99 and 45 mg/liter at 12 and 84 h, respectively. These results indicated that the majority of BACs (about 80%) were degraded within 12 h, while some products of BAC biotransformation and/or accumulating not-easily-degradable organic matter (e.g., cellular decay and lysis products) likely were present after 12 h and observed as residual sCOD. Other previ-

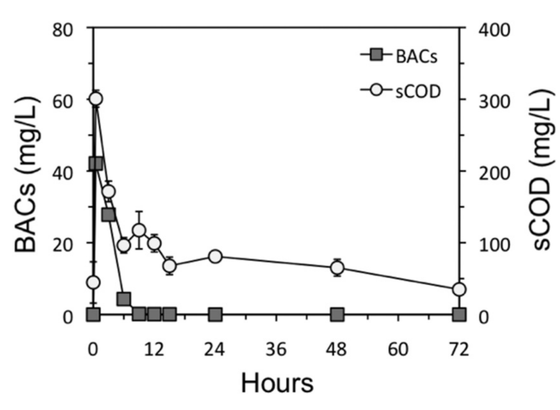

FIG 1 BAC concentration and soluble COD (sCOD) during a feeding cycle. The graph represents a typical feeding cycle of the bioreactor. The BAC supplied to the bioreactor was a 60:40 mixture of C_{12}BDMA-Cl and C_{14}BDMA-Cl; both BAC compounds disappeared at the same time, and the gray squares represent their added concentrations. Measurements were taken in triplicate. Mean values are shown, and error bars represent one standard deviation from the means; errors bars for BAC concentration were too small to show on the graph.

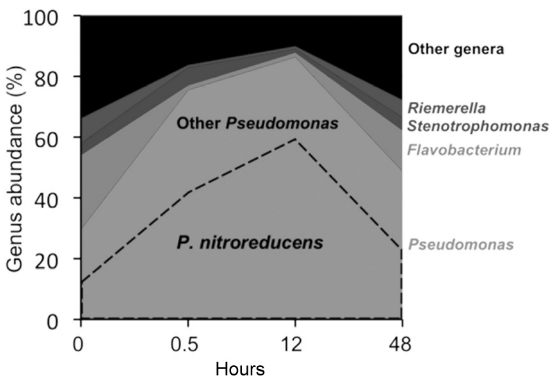

FIG 2 Genus contribution to community metatranscriptome during BAC degradation. The graph represents the fraction of total transcripts that were phylogenetically assigned to major genera (>3% of the total transcripts) based on non-rRNA-encoding reads. Other genera represent the combined fraction of the remaining minor genera. The fractions of total non-rRNA sequences phylogenetically affiliated with *P. nitroreducens* were 12.5%, 42.1%, and 59.7% at 0, 0.5, and 12 h, respectively, and are marked on the graph. Similar results were observed for rRNA-encoding reads (data not shown).

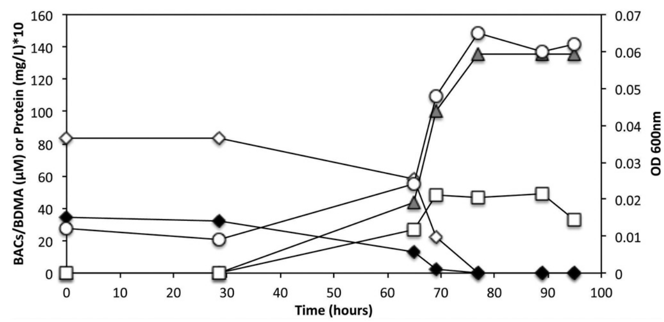

FIG 3 Batch growth of *P. nitroreducens* strain B with BAC as a sole carbon source. Cells were grown in BAC-free medium and subsequently inoculated in half-strength MSB medium supplemented with 50 mg/liter of BAC mixture, as described in Materials and Methods. A control culture containing the same initial biomass but not supplemented with BACs showed no growth based on OD_{600} or protein concentration measurements; uninoculated medium supplemented with BACs showed no decrease in BAC concentration or production of BDMA (negative, abiotic control; see Fig. S2 in the supplemental material). The experiment was performed in triplicate. A representative graph is shown here; the other two replicates showed similar patterns, with the exception that the lag phase before BAC degradation varied (results are available in Fig. S2). ◇, C_{12}BDMA-Cl (μM); ◆, C_{14}BDMA-Cl (μM); ▲, BDMA (μM); □, protein (mg/liter); ○, OD_{600}.

ously reported benzyl-containing BAC intermediates, such as BDMA, benzylmethylamine (BMA), and benzylamine (BA), were not detected during the feeding cycle, presumably due to their fast transformation rates (14), indicating that all BACs were either biodegraded or biotransformed.

Shifts in gene transcript abundance during BAC transformation. The BAC concentration profile observed during a representative feeding cycle guided the selection of sampling time points for metatranscriptomics. Metatranscriptomes at 0.5 h, when BAC concentration was 90% of the total BAC fed to the bioreactor (i.e., BAC biotransformation was under way), and 0, 12, and 48 h, when BAC concentrations were below the detection limit, were determined to analyze shifts in gene transcript abundance in response to BAC feeding (see Table S1 in the supplemental material for statistics for each metatranscriptomic data set). Transcript (cDNA) sequences, excluding identified rRNA reads, were assigned to different genera based on their best BLASTn match to all publicly available complete genomes. Additionally, the relative expression levels of individual genes were calculated based on the number of best BLASTn matches of transcripts against all protein-coding genes assembled from the metagenome of the same community. The comparison of the metatranscriptomic data sets showed that the three biological replicates (from three independent feeding cycles) at 0.5 h clustered closely together based on the relative expression of genera (see Fig. S1A in the supplemental material) and individual gene functions (see Fig. S1B in the supplemental material), revealing high reproducibility between replicates. Therefore, the three metatranscriptomic replicates sampled at 0.5 h were combined for further analysis.

Profiling relative transcript abundance of non-rRNA-encoding genes revealed high abundance of the genus *Pseudomonas* in the metatranscriptomes (86% of total at 12 h), followed by *Stenotrophomonas* (1%), *Flavobacterium* (1%), and *Riemerella* (1%) (Fig. 2). Our previous metagenomic survey (DNA level) of samples taken from the same bioreactor about 3 months earlier than the metatranscriptomes showed the dominance of *Pseudomonas* (57%), followed by *Methylobacterium* (5.6%), *Burkholderia* (2.9%), *Mycobacterium* (2.0%), and *Stenotrophomonas* (1.3%) (17). The metatranscriptomic data revealed high gene expression activity for the most abundant *Pseudomonas* species observed in the metagenomic data and much lower relative expression values (<0.5% of the total community transcriptome) for *Methylobacterium*, *Burkholderia*, and *Mycobacterium* populations. Further analysis revealed substantial shifts in activity of several genera during the feeding cycle. The most pronounced difference was observed for *Pseudomonas*, whose relative gene transcripts made up 30%, 76%, and 86% of the total community at 0, 0.5, and 12 h. Notably, *P. nitroreducens* was highly enriched in the BAC-degrading community based on our previous metagenomic survey (17). Hence, the metatranscriptomic results were consistent with the community structure captured by the metagenomes, albeit with several notable differences among a few relatively abundant populations that showed low transcriptional activity.

BAC transformation by *P. nitroreducens*. As part of the present study, we obtained *P. nitroreducens* isolates from the BAC-fed bioreactor as well as a parallel bioreactor fed BACs plus dextrin and peptone (DPB community) (14) and observed that these isolates can grow on BACs as a sole carbon and energy source by dealkylating the parent molecules and producing stoichiometric quantities of BDMA as a dead-end product (Fig. 3; also see Fig. S2 in the supplemental material). The growth yield of *P. nitroreducens* strain B on the individual constituents of the BAC mixture was independently assessed. Growth yields were 0.128 and 0.172 g of protein/g of substrate for cultures grown on C_{12}BDMA-Cl and C_{14}BDMA-Cl (or 0.233 and 0.313 g of cells/g of substrate, assuming protein constitutes 55% of cell mass). The protein yield appeared to be relatively low compared to the energy contained in BACs, which was consistent with the hypothesis that *P. nitroreducens* can dealkylate BACs and obtain energy from the aldehyde products of BAC dealkylation but not BDMA (the other major product of dealkylation). Growth on dodecanal and tetradecanal aldehydes (the product of BAC dealkylation) as a sole carbon and energy source also was confirmed independently. These results are consistent with those observed in the bioreactor and suggested that *P. nitroreducens* can dealkylate BACs and obtain energy from the aldehyde products of BAC dealkylation.

The genome sequences of two representative *P. nitroreducens* isolates, strains B and DPB, were determined as part of the present study. Querying these genome sequences against the assembled contigs from the metagenome assigned to *P. nitroreducens* showed almost no divergence (>99.9% nucleotide identity). Given that the *P. nitroreducens* population in the bioreactor is highly clonal based on our previous metagenomic study (17), these isolates serve as appropriate genetic representatives of the larger population. Mapping non-rRNA-encoding sequences against available whole-genome sequences revealed that *P. nitroreducens* typically made up more than half of the total transcripts assigned to *Pseudomonas* (Fig. 2). Collectively, these results suggested that *P. nitroreducens* played a key role in community BAC degradation, consistent with our previous DNA-based results (17).

***P. nitroreducens* genes overexpressed upon BAC exposure.** Clustering the 6,621 *P. nitroreducens* protein-coding genes based on their relative transcript abundance identified metabolic functions that were differentially expressed at 0.5 h relative to those at 0, 12, and 48 h ($P < 0.01$ by chi-squared test) (Fig. 4A). These functions were associated with (i) energy generation, e.g., tricarboxylic acid (TCA) cycle, serine-glyoxylate cycle, and ubiquinone menaquinone-cytochrome C reductase; (ii) cell wall biosynthesis and maintenance, e.g., KDO2-lipid A biosynthesis, UDP-*N*-acetylmuramate, and peptidyl-prolyl *cis-trans* isomerase; and (iii) cell division and growth, e.g., ribosomal proteins. The 40 genes with the highest expression levels ($P < 10^{-4}$ by chi-squared test) were related to fatty acid metabolism (e.g., acyl-coenzyme A [CoA] dehydrogenase, aldehyde dehydrogenase, enoyl-CoA hydratase, ketol-acid reductoisomerase, and 3-ketoacyl-CoA thiolase) and energy production through the TCA cycle (e.g., succinate dehydrogenase, aconitate hydratase, enoyl-CoA hydratase, ketol-acid reductoisomerase, nucleoside diphosphate kinase, citrate synthase, and succinyl-CoA ligase) (Fig. 4B). Among the latter genes, a predicted amine oxidase showed about 15-fold greater relative transcript abundance at 0.5 h compared to levels at 0 and 48 h. Amine oxidases have been shown to carry out oxidative deamination reactions in addition to oxidative conversion of amines to aldehydes (28–30); thus, they might be responsible for the first dealkylation step (i.e., cleaving the C_{alkyl}-N bond) of the biochemically characterized BAC degradation pathway.

P. nitroreducens gene transcripts enriched at 0 and 48 h relative to those at 0.5 h ($P < 0.05$ by chi-squared test) included the global sigma factor regulators *rpoS* (4-fold more abundant), *rseA* (5-fold), and *rseB* (3-fold) that typically are overexpressed when cells enter stationary phase (31, 32). The expression of global regulators is consistent with the expected physiological status of *P. nitroreducens* based on the BAC concentration profile during the feeding cycle (Fig. 1).

An amine oxidase mediates BAC biotransformation. To assess the biochemical function of the amine oxidase gene (accession number KJ911918) detected by metatranscriptomics, the gene was PCR amplified, cloned, and overexpressed in *E. coli* Rosetta 2(DE3)/pET-KJ911918, and the BAC removal activity of the transformant was compared to that of *P. nitroreducens* strain B, which encodes the native amine oxidase. Cell extracts of the *E. coli* clone and *P. nitroreducens* strain B were dialyzed, and crude cell extract activity was measured with and without addition of flavin adenine dinucleotide (FAD) using C_{12}BDMA-Cl or C_{14}BDMA-Cl as the substrate. Negative controls without cofactors, *E. coli* Rosetta 2(DE3) containing an empty pET vector, or *P. nitroreducens* strain B grown on glucose (no BACs) showed no detectable activity. Consistent with other flavoprotein amine oxidases (33), the cloned amine oxidase required FAD, produced stoichiometric amounts of BDMA (see Fig. S3 in the supplemental material), and was selective toward C_{12}BDMA-Cl (Table 2). The substrate preference was similar in the clone and the wild type, indicating that the amine oxidase gene encodes an enzyme responsible for BAC dealkylation in *P. nitroreducens* strain B. However, the enzyme from *P. nitroreducens* cell extracts showed slightly higher activity for C_{12}BDMA-Cl than the enzyme in the clone (Table 2). This result could be attributable to experimental error, lack of appropriate accessory proteins in the cell extracts of the *E. coli* clone, or the presence of an additional BAC-dealkylating enzyme in *P. nitroreducens* strain B (although we were unable to detect other candidate enzymes based on bioinformatics sequence analysis). Further work is required to provide more detailed resolution of the biochemistry of the reaction.

Activities of other members of the community. Metabolic responses of other (non-*P. nitroreducens*) community members also were characterized during the feeding cycle. The metatranscriptomic profiles at 0.5 h were distinguishable from those at other time points (see Fig. S4A in the supplemental material) and showed increased expression of genes predicted to be associated with (i) the biodegradation of benzoate (i.e., the by-product of BDMA metabolism), such as benzoate dioxygenase (*benABC*), (ii) energy production (serine-glyoxylate cycle), and (iii) cell division/cycle (ribosome protein and biosynthesis). The majority of these transcripts, including those related to benzyl-containing compound metabolism, were phylogenetically affiliated with *Pseudomonas* species (83% on average), particularly *P. putida* (31%) and *P. entomophila* (15%) (see Fig. S4B). Therefore, these results indicated that the benzyl compounds produced from the dealkylation of BACs by *P. nitroreducens* (14) were predominantly metabolized by *P. putida* and *P. entomophila* (Fig. 5).

Our previous metagenomic study revealed three mobile genetic elements carrying genes predicted to be associated with antibiotic and/or BAC resistance (but not biodegradation), all of which had been rapidly and reproducibly selected by BAC exposure (17). One of these genetic elements encoded four predicted efflux pump systems potentially capable of exporting a wide range of toxic compounds, including two small multidrug resistance (SMR) family systems (*sugE*), one resistance nodulation division (RND) family system, and one ABC transporter system (see Fig. S5A in the supplemental material). Further study indicated that these efflux pumps confer increased resistance to BACs as well as several antibiotics (7). In the present work, metatranscriptomics revealed significantly increased expression ($P < 0.01$ by chi-squared test) of these four efflux pump systems at 0.5 h compared to results at 0, 12, and 48 h (see Fig. S5A). This genomic island also was present in other members of the bioreactor community, likely as part of a conjugative plasmid acquired horizontally, and organisms isolated from clinical samples (see Fig. S5B and C). Together, these results indicated that the genes on this putative mobile element play a significant role in coping with toxicity by regulating intracellular BAC concentrations in both BAC-degrading and nondegrading members of the community.

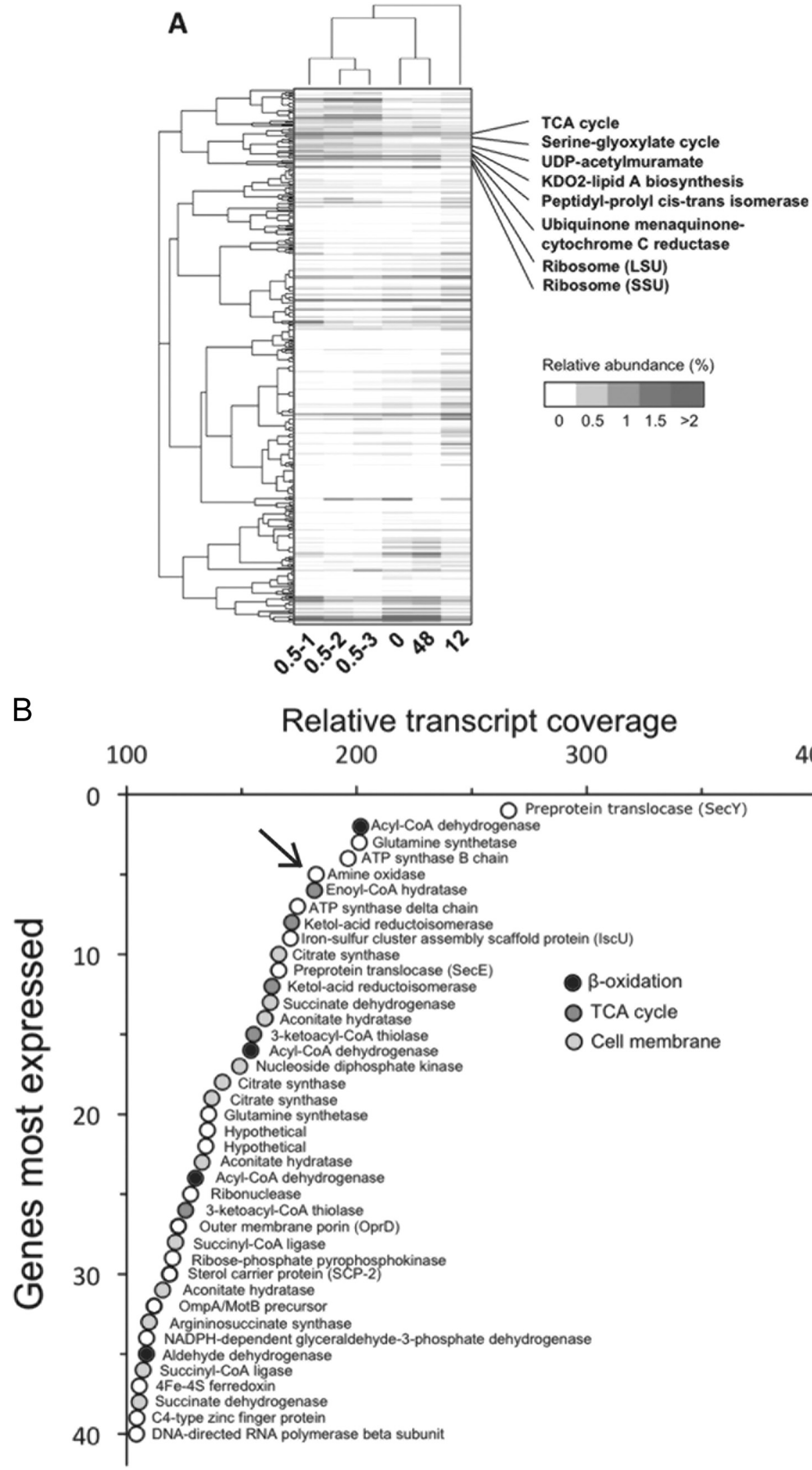

FIG 4 Gene functions expressed by *P. nitroreducens* during BAC degradation. (A) Clustering of the relative abundance of SEED subsystems. Hierarchical clustering was carried out using the Spearman rank correlation metric and complete linkage as implemented in Cluster 3.0 software (41). Selected SEED subsystems significantly overexpressed at 0.5 h as opposed to at 0, 12, and 48 h ($P < 0.01$ by chi-squared test) are denoted on the graph. (B) List of the genes that were most overexpressed at 0.5 h ($P < 10^{-4}$ by chi-squared test), excluding ribosomal protein genes. The BAC-dealkylating amine oxidase is denoted with the arrow.

TABLE 2 Specific enzyme activity of amine oxidase with dialyzed cell extracts

Strain	Substrate activity[a] (nmol substrate/mg of protein/min)	
	C_{12}BDMA-Cl	C_{14}BDMA-Cl
Rosetta 2(DE3)/pET-KJ911918	ND	ND
Rosetta 2(DE3)/pET-KJ911918 with no cofactor	ND	ND
Rosetta 2(DE3)/pET-KJ911918 with FAD as a cofactor	19.2 ± 4.3	6.6 ± 1.5
P. nitroreducens strain B with FAD as a cofactor (grown on glucose)	ND	ND
P. nitroreducens strain B with no cofactor (grown on BACs)	ND	ND
P. nitroreducens strain B (grown on BACs) with FAD as a cofactor	36.8 ± 3.5	6.1 ± 1.3

[a] Measurements were performed in duplicate, and average values and variations around the means are shown. ND, not detected.

DISCUSSION

In this study, we provided the first metatranscriptomic view of a whole microbial community degrading BACs under aerobic batch-fed conditions and obtained insights into key community members, their cooperative interactions, and genes involved in BAC detoxification and biodegradation. BACs completely disappeared within 12 h (Fig. 1), and *P. nitroreducens* played a key role in BAC degradation based on high gene expression levels (e.g., Fig. 2 and 4) and encoded at least one key enzyme responsible for BAC dealkylation, amine oxidase. Since the dealkylated BDMA is about 500 times less toxic than BACs (14), the dealkylation of BACs is also an important detoxification process that presumably provides significant benefits to other community members. Detoxification of BACs or toxic intermediates also is expected to be mediated, at least in part, by the activity of efflux pump systems on a genomic island that was shared by several non-BAC-degrading members of the community (see Fig. S5 in the supplemental material) (7). Community metatranscriptomic data, as well as metabolite analysis of cultures of isolates grown on BACs, indicated that *P. nitroreducens* strain B cannot metabolize BDMA and, after dealkylation of BACs, appears to generate energy by β-oxidation of the alkyl chain and the TCA cycle (Fig. 3 and 4 and Table 2). Based on the assignment of transcripts to individual community members, benzyl-containing compounds (intermediates of BDMA biodegradation) were metabolized predominantly by other *Pseudomonas* species, such as *P. putida* and *P. entomophila* (see Fig. S4). Collectively, the metatranscriptomic, metabolite, and biochemical data indicated that the microbial community robustly and rapidly (e.g., within 12 h) biodegraded BACs and that cross-feeding among key community members facilitates the biodegradation (Fig. 5).

Our results indicated that an amine oxidase enzyme was responsible for BAC dealkylation by *P. nitroreducens*. Amine oxidases are capable of oxidizing a variety of amines and are divided into copper- and flavin-containing classes; both classes have been shown to catalyze deamination reactions as well (28, 29). Recently, an NADPH- and FAD-containing monooxygenase was reported to cleave quaternary ammonium compounds, specifically tetradecyltrimethylammonium bromide (34). Our results show that this gene is not present in the *P. nitroreducens* genomes or the metagenome/metatranscriptome reported here. Instead, the *P. nitroreducens* isolate appears to encode an unrelated amine oxidase enzyme (see Fig. S6 in the supplemental material) that is responsible for BAC deamination. Pseudooxynicotine amine oxidase (AFD54463.1) from *Pseudomonas* sp. strain HZN6 was the closest (42% amino acid identity) biochemically characterized match to the candidate amine oxidase encoded by genes of *P. nitroreducens* strain B. Pseudooxynicotine amine oxidase requires FAD and oxygen to cleave the carbon-nitrogen bond and produces an aldehyde, methylamine, and hydrogen peroxide (35). Both BAC-dealkylating and pseudooxynicotine amine oxidase have Rossmann-like FAD binding sites in common, consistent with the requirement for an FAD cofactor for activity in dialyzed cell extracts. The relatively low sequence identity between the two enzymes is consistent with the different substrate specificity (i.e., against BACs) for the enzyme reported here. Hence, bioinformatics analysis and results from previous literature are consistent with results reported here that a bioinformatically inferred amine oxidase can catalyze the dealkylation of BACs. We also propose that gene KJ911918 be named BAC-dealkylating amine oxidase.

It should be noted that additional BAC-degrading organisms and genes likely exist in nature and may even be present within our bioreactor, as the specific activity of the cloned amine oxidase relative to the activity of the native amine oxidase against the two BAC compounds used in the feed is substantially lower (Table 2). We reported on only one such biodegradation pathway and a BAC detoxification mechanism identified by a combination of bioinformatics and metatranscriptomics techniques here. Thus, our study represents an example of how omics methodologies can be used to pinpoint candidate biodegradation genes among the thousands encoded by a natural (or enriched) community and guide appropriate genetic experiments to test predictions of the bioinformatics analysis of omics data. Such studies will provide a more complete picture of the microbial biotransformations of BACs and other biocides in nature.

Several additional *Pseudomonas* species present in the community may be able to metabolize intact BACs based on previous

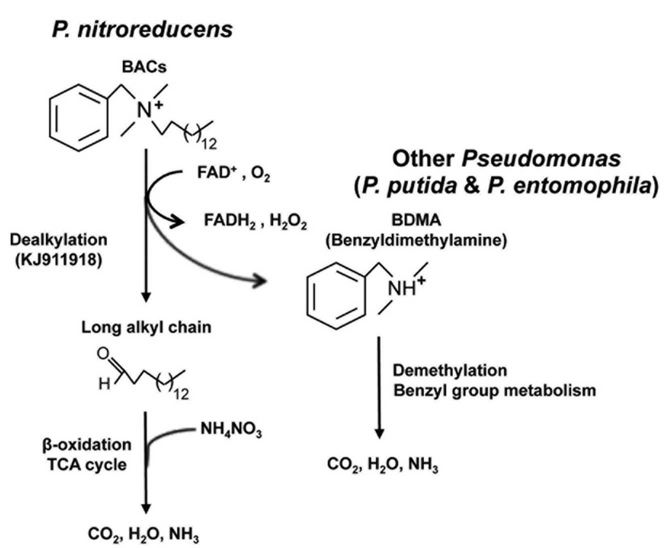

FIG 5 Model of BAC (C_{14}BDMA-Cl) metabolism by the microbial community in the B bioreactor. The genes involved in BAC dealkylation and β-oxidation based on bioinformatics and/or genetic analyses are shown.

results with *P. putida* isolates (14) and our own observations. Nevertheless, *P. nitroreducens* dominated the community described in this study as well as other communities established from the same inoculum with different BAC concentrations or feeding regimens (17). The *P. nitroreducens* genomes carried multiple copies of aldehyde dehydrogenase and alcohol dehydrogenase genes, unlike other *Pseudomonas* genomes of the community, and several of them were highly expressed at 0.5 h (Fig. 4B). Aldehyde dehydrogenase and alcohol dehydrogenase potentially are related to alkyl chain metabolism (e.g., β-oxidation and fatty acid biosynthesis pathway) and likely confer a competitive advantage to *P. nitroreducens* in metabolizing the alkyl chain by-products of BAC dealkylation. Further, cell membrane-associated genes assigned to *P. nitroreducens*, such as outer membrane porin/lipoprotein, SCP-2 sterol transfer protein, and 3-oxoacyl-(acyl carrier protein) reductase, were among the top 40 most expressed genes at 0.5 h (Fig. 4B). Although the specific function of the outer membrane lipoprotein remains unclear, a previous study reported increased expression of this gene in response to QACs and suggested that it functions either as an efflux pump or to physically enhance membrane integrity against QACs (36). Sterol carrier proteins transfer steroids across cellular membranes (37), and enoyl-acyl carrier reductase is involved in the fatty acid elongation cycle (38); thus, both genes modulate membrane fluidity, permeability, and thickness and could play a role in alleviating BAC toxicity by enhancing cell membrane integrity. Cell membrane biosynthesis represents an important cellular response to BACs, which are membrane disruption agents (39, 40). Hence, the genes identified here might contribute to the fitness advantage of *P. nitroreducens* related to maintaining cell membrane integrity in the presence of BAC toxicity, although this hypothesis awaits experimental verification.

The organisms (e.g., *P. nitroreducens*) and sequences, specifically the amine oxidase gene, reported here will aid the development of culture-independent tests to assess the abundance and activity of microorganisms controlling the fate of BACs under aerobic conditions in natural or engineered systems, such as WWTPs. Furthermore, the microbial consortia described in this study efficiently removed (biodegraded) BACs in a laboratory-scale bioreactor and could inform the future design of larger engineered systems tailored to remove BACs from municipal and industrial waste streams. Therefore, our findings have important implications not only for the (appropriate) use of disinfectants, as they illustrate the potential for BACs to dramatically alter the structure of nontarget microbial communities, but also for assessing, predicting, and optimizing biologically engineered processes to promote detoxification by revealing key BAC-degrading microbes and enzymatic functions to ensure public and environmental health.

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We have no conflicts of interest to declare.

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