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Partnership of *Arthrobacter* and *Pimelobacter* in Aerobic Degradation of Sulfadiazine Revealed by Metagenomics Analysis and Isolation

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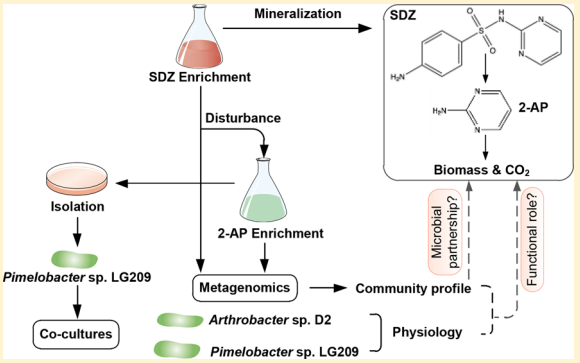
Supporting Information

ABSTRACT: In this study, metagenomic analyses were combined with cultivation-based techniques as a nested approach to identify functionally significant bacteria for sulfadiazine biodegradation within enrichment communities. The metagenomic investigations indicated that our previously isolated sulfadiazine degrader, *Arthrobacter* sp. D2, and another *Pimelobacter* bacterium concomitantly occurred as most abundant members in the community of an enrichment culture that performed complete sulfadiazine mineralization for over two years. Responses of the enriched populations to sole carbon source alternation further suggested the ability of this *Pimelobacter* member to grow on 2-aminopyrimidine, the most prominent intermediate metabolite of sulfadiazine. Taking advantage of this propensity, additional cultivation procedures have enabled the successful isolation of *Pimelobacter* sp. LG209, whose genomic sequences exactly matched that of the dominant *Pimelobacter* bacterium in the sulfadiazine enrichment culture. Integration of metagenomic investigations with the physiological characteristics of the isolates conclusively demonstrated that the sulfadiazine mineralization in a long-running enrichment culture was prominently mediated by primary sulfadiazine-degrading specialist strain *Arthrobacter* sp. D2 in association with the 2-aminopyrimidine-degrading partner strain *Pimelobacter* sp. LG209. Here, we provided the first mechanistic insight into microbial interactions in steady sulfadiazine mineralization processes, which will help develop appropriate bioremediation strategies for sulfadiazine-contaminated hotspot sites.



INTRODUCTION

Sulfonamides were one of the most commonly purchased antibiotics on the market, mainly for the intensification of food animal production in agriculture.[1] Survey data reported that sulfonamides accounted for about 11 to 23% of the annual sales of veterinary antibiotics in 25 European countries in 2011.[2,3] Sulfonamides excreted from animals or human were generally insufficiently treated by wastewater treatment plants,[4−7] and sulfonamide residues were ubiquitously found in various environmental compartments (e.g., surface water,[8−10] soil,[11−13] and groundwater[14−17]) in nanograms per liter to micrograms per liter concentrations. The selection pressures due to the presence of sulfonamide antibiotics in the open environment may promote the dissemination of sulfonamide resistant genes in clinically relevant pathogens[18] and potentially threaten the therapy effectiveness of sulfonamides. The implementation of sulfonamide degraders in sulfonamide-contaminated hotspot sites (e.g., discharge points for pharmaceutical wastewater, livestock farm wastewater and hospital wastewater) through biological wastewater treatment is a pragmatic remediation method. This method can combat sulfonamide resistance by directly reducing sulfonamide concentrations in the pollution sources and thus mitigate sulfonamide exposure level to the pathogenic microorganisms. Since these sulfonamide degraders are also sulfonamide-resistant microorganisms, to avoid their distribution from the biological treatment reactors (such as activated sludge reactors) to the open environment, treatment should be carried out within enclosed engineered systems instead of natural environment which is more difficult to control. In addition, postcontrol strategies like membrane separation of biologically treated effluent coupled with incineration of the biomass sludge should be applied.

Preliminary attempts to identify the key degraders within sulfonamide-degrading consortia have seen variable selections on disparate species.[19−23] This could mainly due to perturbations imposed by different carbon source amendments

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(e.g., sodium acetate or yeast extract) during sulfonamide exposures. Enrichment culturing strategies featuring sulfonamide as the sole carbon source should facilitate more explicit interpretations of the community responses that were actually driven by sulfonamide catabolic activities. Particularly, a few studies[24,25] examined the overall microbial populations that were capable of subsisting on sulfonamides, only using 16S rRNA-based molecular methods. The function of individual microorganisms and ecology of the community, however, are difficult to be fully understood and characterized just based on phylogenetic classifications.

Cultivation-based techniques have already taken a prominent leap forward through efforts in isolation of the sulfonamide-degrading specialist bacteria. A diverse array of bacterial isolates in the phyla *Proteobacteria*[26−30] and *Actinobacteria*[31−33] have been demonstrated the capabilities to grow on different classes of sulfonamides. Despite the sulfonamide catabolic efficiency measured in particular single isolate were promising under microcosm-scale laboratory experiments, addition of sulfonamide-degrading bacteria in near-field conditions failed to increase the sulfonamide biodegradation rates,[34,35] most likely owing to the poor survival or low activity of the inoculated specialists in the natural environment. Improved understanding on how the key degraders establish and maintain their memberships with other participating bacteria during the biodegradation processes can aid further in sufficiently exploiting the catabolic activities of the sulfonamide-degrading isolates.

Driven by the hypothesis that structurally similar sulfonamides being sole carbon source would lead to the enrichment of similar key degraders possessing prevalent sulfonamide degradation strategy (such as *ipso*-hydroxylation coupling subsequent fragmentation of the mother molecule[36]), we established the enrichment cultures of two frequently encountered sulfonamides (sulfadiazine (SDZ) and sulfamethoxazole (SMX)). Metagenomic approaches can bypass the cultivation needs, and have been demonstrated highly informative in profiling microbial structures of natural[37,38] and engineered[39,40] microbiomes. With an aim to compare these two microbial communities, two metagenomic analysis strategies, i.e. the reconstructed 16S rRNA gene analyses and binned genome analyses, were used to catalog species-level diversities of these two long-running sulfonamide enrichment cultures. In addition, our previous study characterized the SDZ enrichment community at day 285 with 16S rRNA PCR cloning approach, and isolated two *Arthrobacter* strains, with strain D2 exhibiting distinct sulfadiazine degradation abilities.[41] Though the SDZ degradation processes by isolate D2 have been extensively documented in our previous study, its role in the ecological network of microbial community of SDZ enrichment culture has yet to be elucidated. Here, the SDZ enrichment culture was specifically used as a model community to illustrate how metagenomic analyses can be incorporated with pure culture physiological characterizations to reveal the roles of the isolated degrader, and to understand its interactions with other community members in the SDZ biodegradation process.

MATERIAL AND METHODS

Culture Enrichment and Metagenomic Sequencing and Assembly. Two sulfonamide-degrading cultures were inoculated with the same activated sludge sampled from a local sewage treatment plant (Shek Wu Hui Sewage Treatment Work, Hong Kong). These two cultures were maintained in 500 mL serum bottles, in a semibatch mode, with mineral salts medium (MSM)[41] containing SDZ or SMX as sole carbon source for total 812 days (Figure S1). To select 2-aminopyrimidine (2-AP, the most prominent intermediate during SDZ mineralization by SDZ enrichment culture) degraders, we therefore established the 2-AP enrichment seeded with subculture of SDZ enrichment (at day 767). A 20 mL portion the SDZ enrichment cultures was transferred into 180 mL fresh MSM supplied with 100 ppm of SDZ and 100 ppm 2-AP (SDZ +2-AP enrichment). After three draw-and-fill cycles (a total of 23 days), the carbon source was changed to sole 2-AP with 200 ppm concentration and was maintained for another three cycles. At the sampling time (SDZ enrichment at day 812, SMX enrichment at day 812, SDZ+2-AP enrichment at day 23, and 2-AP enrichment at day 45), approximately 100 mL of enrichment liquid was taken and subsequently subjected to DNA extraction using FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH). Four genomic DNA samples (SDZ enrichment, SMX enrichment, SDZ+2-AP enrichment, and 2-AP enrichment) were sent to BGI (Shenzhen, China) for paired-end library construction (insert size of 350 bp) and sequencing via Illumina HiSeq-2500 platform. The yield 150 bp paired-end reads were filtered and assembled independently with CLC Genomics Workbench software (CLC bio, Denmark). Only the contigs with length over than 500 bp were included for downstream analyses.

Community Profiling and Detection of Sulfonamide Resistance Genes. Initially, MetaPhlAn2 (v.2.6.0) was applied to assess the overall taxonomic compositions of the enrichment communities by mapping total raw reads to around 1 million taxonomy-specific marker genes.[42] Since a majority of the clean reads were assembled into contigs, we also used the protein-coding sequences located on the contigs to further characterize the microbial community compositions. All assembled contigs were predicted into open reading frames (ORFs) by Prodigal[43] and subjected to homologous screening against the proteins in the NCBI-RefSeq database using RAPSearch2[44] (E-value < 10^{-5}). The taxonomy of each ORF was assigned by MEGAN (v 5.10.3)[45] using the default parameters of the lowest common ancestor algorithm. Reconstruction of the near full-length 16S rRNA gene sequences from metagenomic data sets were performed by EMIRGE algorithm[46] with 80 iterations. DECIPHER[47] was used to detect the chimera among EMIRGE reconstructed sequences. Phylogenetic classifications of the resulting sequences were determined by two approaches: taxonomic assignments of SILVA (SINA online)[48] with 95% minimum identity and 10 neighbors per query, and online nucleotide BLAST against NCBI nr database for query sequences that were unclassified by SILVA database. The relative abundance of annotated query sequences was derived from EMIRGE algorithm on the basis of prior probabilities of the read coverage. For EMIRGE-reconstructed 16S rRNA genes, similarity percentage analyses (SIMPER) was used to identify taxa contributing to dissimilarities among two enrichment groups through Bray−Curtis distances. The detection of sulfonamide resistance genes in the raw data sets of enrichment cultures was performed via an online detection pipeline for antibiotic resistance genes (available at http://smile.hku.hk/SARGs).[49]

Population Genome Binning, Annotation, and Analysis. Three metagenomes were subjected to coassembly for binning of population genomes. Following the reported

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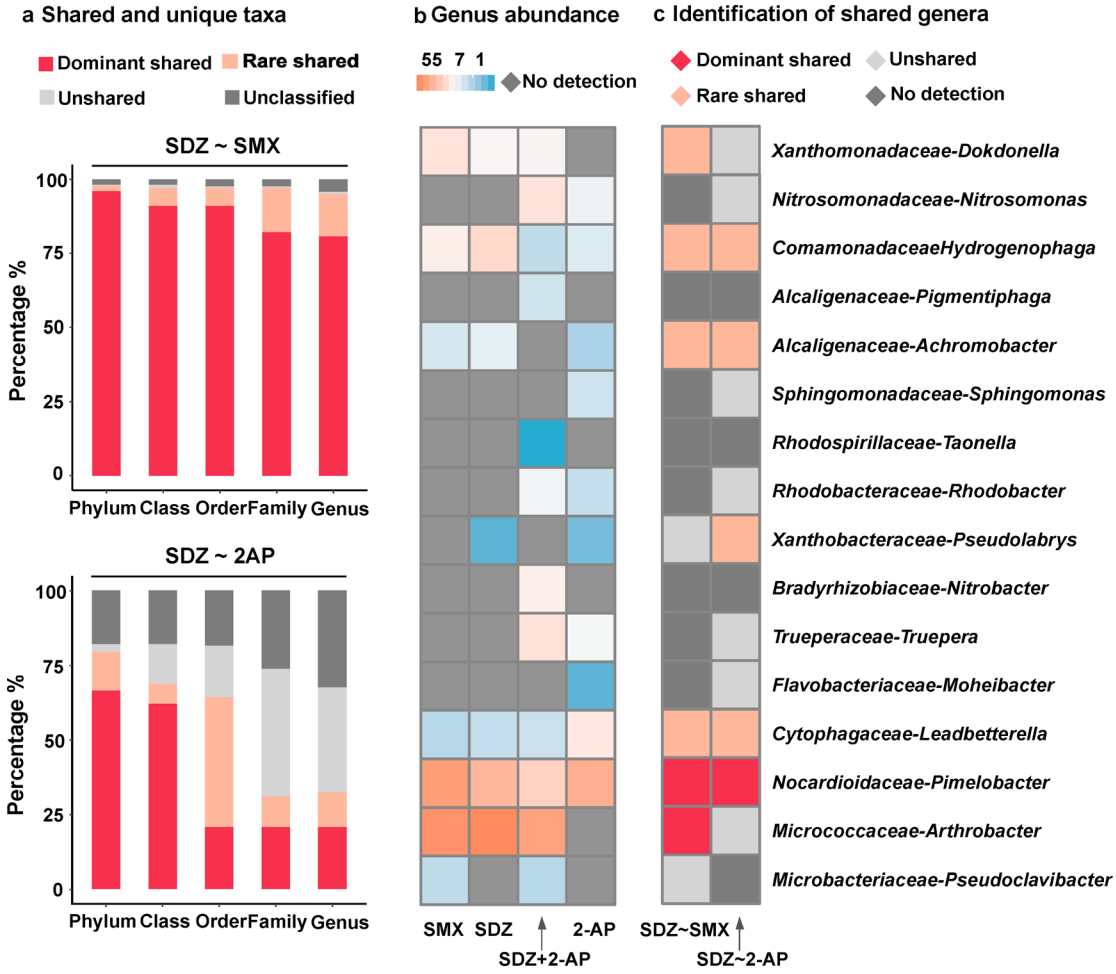

Figure 1. Taxonomic compositions across four enrichment cultures based on EMIRGE-reconstructed 16s rRNA gene sequences. (a) Shared and unique taxa of SMX and 2-AP enrichments in relative to SDZ enrichment. (b) Genus abundance within each enrichment community. (c) Identification of genera as dominant shared (present at >5% relative abundance in both samples), rare shared (present at <5% relative abundance in both samples), or unshared.

mmgenome workflow,[50] population genomes were reconstructed based on the differences in GC content, tetranucleotide frequencies and sample coverage. Genome quality assessment was calculated with CheckM (v1.0.5).[51] Binned genome sequences were submitted to IMG/ER[52] for annotation and data sharing (see Table S1 for accession numbers). The 16S rRNA genes presented in the genome were identified through RNAmmer.[53] Genome phylogeny was evaluated by a genome tree which was constructed by a concatenated set of 16 benchmarked ribosomal proteins[54] and visualized in iTOL.[55] The 2563 reference genomes included in the genome tree were downloaded from public data sets including NCBI, IMG/ER, and ggKbase.

Pure Culture Isolation and Batch Experiments. Dilutions of small volume of the 2-AP enrichment cultures were spread on mineral salts agar plates supplemented with 200 micrograms per liter (ppm) 2-AP as sole carbon source. After 1 week, single colonies grown on the plate corresponding to the most diluted fraction were picked, followed by restreaking of selected single colonies on corresponding plates, and the purification procedures were repeated for five times. 16S rRNA genes in the purified colonies were amplified by polymerase chain reaction (PCR) using the universal primes 27F and 1492R, and were sent to BGI (Shenzhen, China) for Sanger sequencing. The extraction and sequencing of the genomic sample of the identified strain LG209 were performed as the same as the metagenomic samples. Assembled draft genome sequences of strain LG209 were deposited in GenBank under the accession numbers NHTS00000000.

The activity of the new strain LG209 on degrading SDZ and 2-AP were tested in 100-mL serum bottles in duplicates under three different nutrient conditions: (1) MSM supplemented with 200 ppm of SDZ or 2-AP; (2) MSM supplemented with 200 ppm of SDZ or 2-AP together with 2000 ppm glucose; (3) LB medium amended with 50 ppm of SDZ or 2-AP. Liquids were taken at specified intervals. The dissipation of mother compound and generation of metabolites were measured by ultraperformance liquid chromatograph coupling double mass detector (UPLC-MS/MS) using the detection methods we reported before.[41]

RESULTS AND DISCUSSION

Enrichment of SDZ-Degrading Consortia. The SDZ-degrading culture examined here was derived from the activated sludge sampled from a local municipal wastewater treatment plant (Shek Wu Hui Sewage Treatment Work, Hong Kong). Consecutive transfers of portions (10−20%) of these cultures were performed over 2 years in MSM supplemented with SDZ

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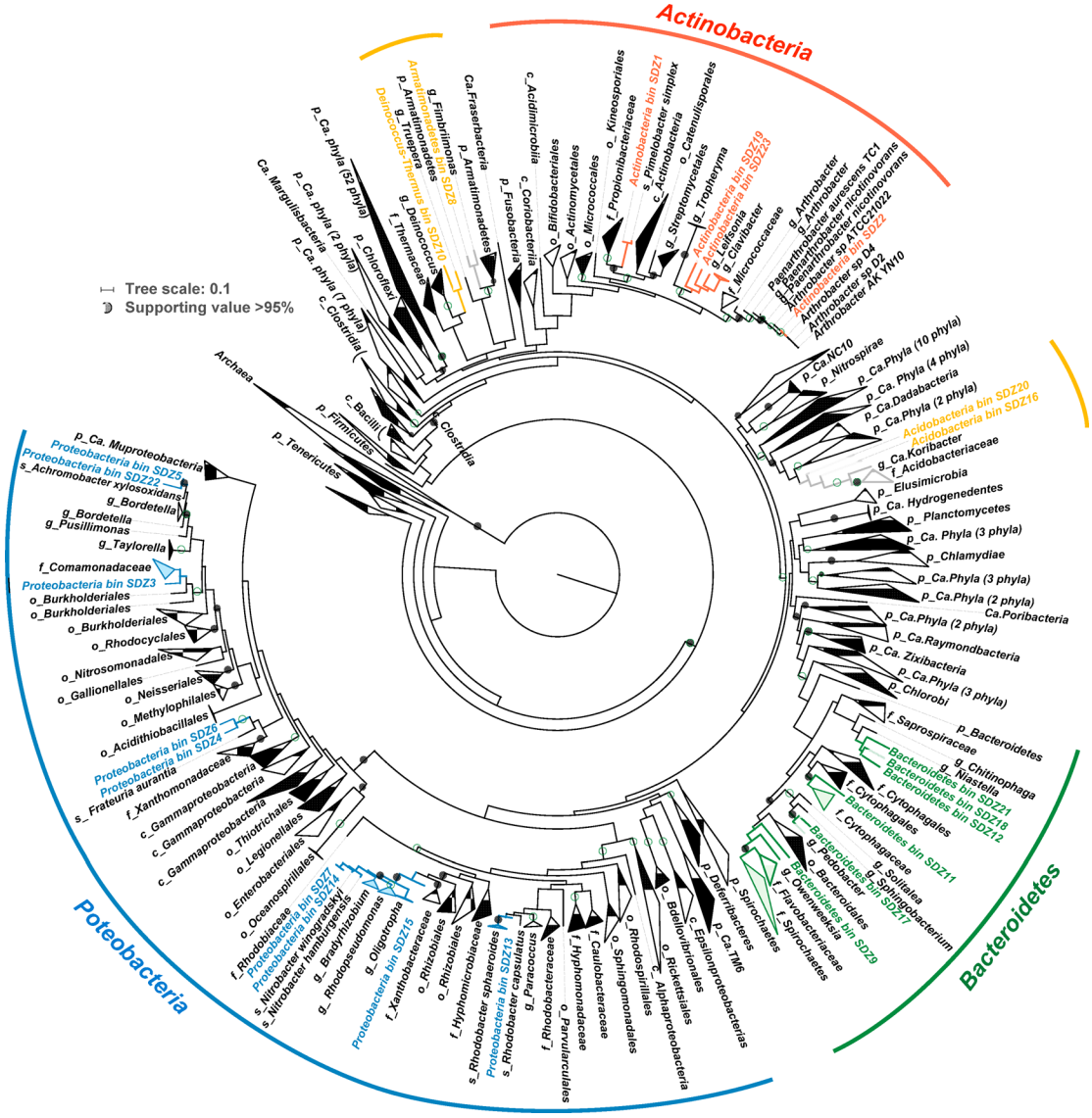

Figure 2. Phylogenetic placements of 23 population genomes in relative to 2563 genomes using 16 concatenated ribosomal proteins.

as the sole carbon source in incrementally increasing concentrations (50, 100, and 200 mg/L). SDZ degradation activity can be recovered repeatedly from the dilutions of these enrichment cultures, as indicated by the periodically stable reduction of the total organic carbon (TOC) in the medium (Figure S1), suggesting sufficient enrichment of SDZ-degrading consortia. In order to address the roles of uncharacterized or uncultivated microorganisms presented in the long-running enrichment culture, biomass from SDZ enrichment at day 812 were sampled for metagenomic analyses. At the sampling point, SDZ biodegradation occurred at degradation rate of 0.12 mg $L^{-1}h^{-1}$ (half-life time was 5.84 h, Figure S2). Meanwhile the corresponding TOC reduction rate was 92% for SDZ enrichment culture, respectively, achieving almost complete mineralization.

To understand the microbial interactions underpinning sulfonamide biodegradation processes, we further detected important intermediates during SDZ degradation by SDZ enrichment culture using UPLC-MS/MS as we previously reported.[41] Consistent production of 2-aminopyrimidine (2-AP) was observed as the most prominent intermediate before complete TOC reduction in SDZ enrichment culture. It is notable that a majority of the sulfonamide-degrading isolates have shown their limited abilities to the degradation of the heterocyclic moiety of sulfonamides;[27,28,31,32] only a part of the organic carbon derived from sulfonamides can be mineralized by these isolates to CO_2 or biomass. Thus, the nearly complete SDZ mineralization observed in the enrichment culture were presumably mediated either by primary SDZ degraders in association with 2-AP-degrading partner populations, or, contrastingly, by single microorganism that occupy the ecological niches required for complete SDZ mineralization. To track the abundance changes of microbial populations in response to sole carbon source alternation, metagenomic samples from this enrichment were collected at two time points (day 23, SDZ+2-AP enrichment; day 45, 2-AP enrichment) when substantial TOC reductions were demonstrated (Figure S1).

Microbial Community Overview. Four metagenomic data sets (28.7 Gb total raw reads) were individually assembled and annotated for taxonomic profiling. On average, 90.2% of the raw reads from each metagenome could be mapped onto

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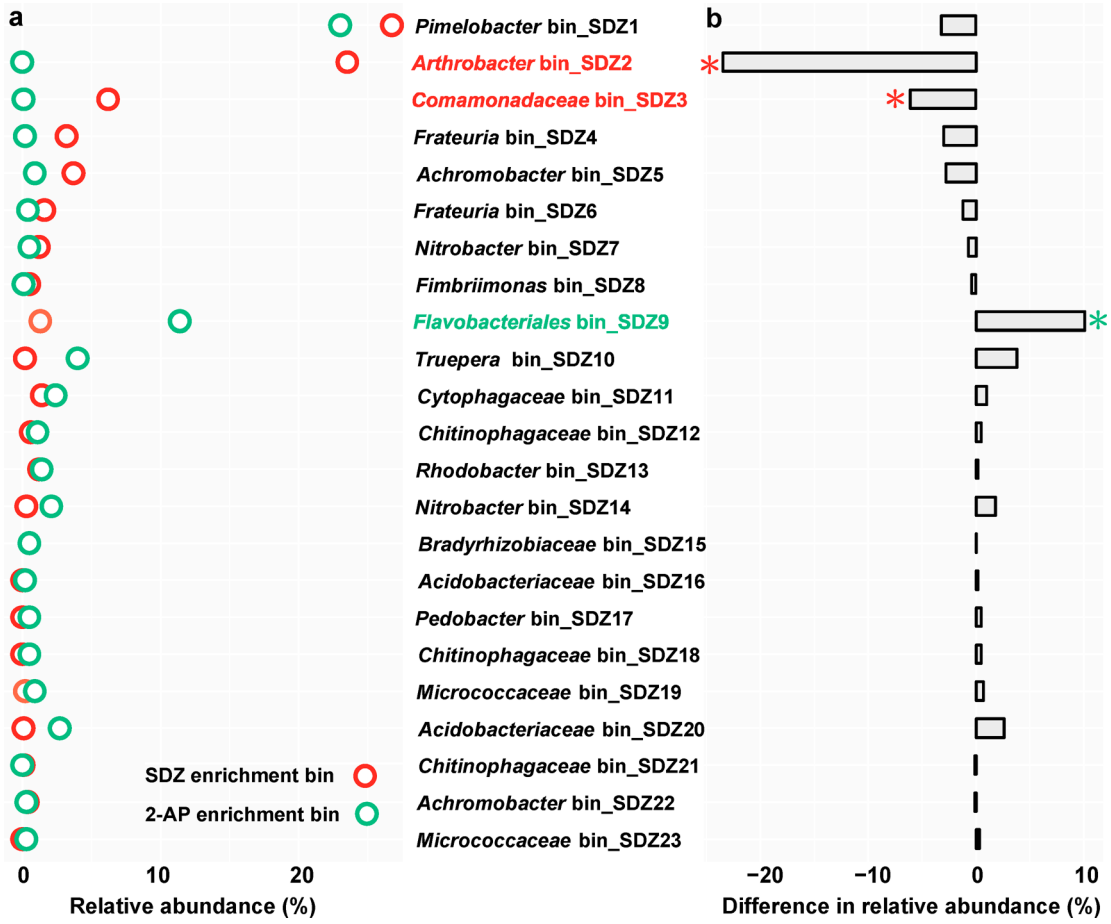

Figure 3. (a) Relative abundance of recovered population genomes in SDZ and 2-AP enrichment communities. Relative abundance was estimated based on average coverage of the binned contigs. (b) Difference in genome relative abundance between SDZ and 2-AP enrichment communities. Three most significant contributors to the abundance difference were identified by SIMPER analysis and were highlighted with ∗ symbols in red (overrepresented in SDZ enrichment) or green (overrepresented in 2-AP enrichment).

the contigs from the assembly, indicating the efficient usage of the raw reads. The microbial communities of four enrichments were primarily evaluated by read-based taxonomic assignments via MetaPhlAn2 and phylogenic classifications of total functional proteins via MEGAN. Both approaches identified bacteria as the dominating microorganisms across all samples, with little eukaryotic (<5.2%) and archaeal (<0.1%) detected (Table S2). Representative bacterial populations were further determined by EMIRGE-reconstructed 16S rRNA gene sequences. In addition, we also checked the sulfonamide resistance gene abundance in the raw data sets of SDZ enrichment culture. The sulfonamide resistance gene abundance were found as 1.1 copy per cell in SDZ enrichment culture, largely increased during the enrichment process compared with the level in the seed sludge (0.05 copy per cell).

Not surprisingly, the SDZ enrichment community showed high similarity to another sulfonamide enrichment community (SMX enrichment), at genus level, shared taxa by these two communities comprised over 90% of the total communities (Figure 1a). Specifically, the relative abundance of EMIRGE-reconstructed 16S rRNA genes revealed that *Actinobacteria* was by far the most abundant phylum (80.9%) in the community of SDZ enrichment (Table S3), among which the genera *Arthrobacter* (59.9%) and *Pimelobacter* (18.2%) were notably dominant (Figure 1b). The other genera (including *Dokdonella*, *Hydrogenophaga*, *Achromobacter*, and *Leadbetterella*, Figure 1c) constituted a small fraction of SDZ enrichment, accounting for 15.1% in the SDZ enrichment community.

On the other hand, occurring as microbial responses to sole carbon source alternation, evidently differentiated community structures were observed in SDZ and 2-AP enrichment cultures, though the inoculum for the 2-AP enrichment was derived from the SDZ enrichment. The *Pimelobacter* similarly dominated in the 2-AP enrichment community (23.3%) and the relative abundance of *Arthrobacter* in the 2-AP enrichment quickly declined from 32.7% (day 23) to 0 (day 45) over the period of 22 days. To identify the contribution of certain taxa to the differences between these two communities, a similarity percentage (SIMPER) analysis[56] was performed (Tables S4 and S5). Intriguingly, the observed compositional variations between SDZ and 2-AP enrichments were primarily driven by the changes of *Arthrobacter* populations in the communities (>44% of variation at genus level). The absence of *Arthrobacter* populations in the 2-AP enrichment may result from its inability to use 2-AP as carbon source.

***Arthrobacter* and *Pimelobacter* Populations in the SDZ-Degrading Consortia.** 16S rRNA genes may not allow enough resolution at genome level for the analyses of functional roles of individual members in the consortia. To investigate the roles of individual members in SDZ biodegradation, genome-centric exploration of the microbial communities were further performed for metagenomes of SDZ enrichment, SDZ+2-AP

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enrichment, and 2-AP enrichment. The three metagenomic data sets were thus subjected to combined assembly and differential coverage-based binning, resulting in the recovery of 23 bacterial population genomes (Table S1 and Figure S3) that were nearly complete (72−98% completeness and 0−8% contaminations). According to the percentage of reads mapping,[39] these 23 draft genomes accounted for the majority of the microbial communities (74.7%, 61.9%, and 56.2% for SDZ, SDZ-2-AP, and 2-AP data sets, respectively). A genome tree, inferred from 16 previously benchmarked ribosomal proteins,[54] showed that the recovered 23 population genomes represented organisms of the phyla *Proteobacteria* (nine genomes), *Bacteroidetes* (six), *Actinobacteria* (four), *Acidobacteria* (two), *Armatimonadetes* (one), and *Deinococcus-Thermus* (one) (Figure 2 and Table S6).

Similar to EMIRGE 16S rRNA gene analyses, *Actinobacteria* genomes constituted the most abundant fraction in SDZ-degrading consortia. Specifically, *Pimelobacter* bin_SDZ1 and *Arthrobacter* bin_SDZ2 were estimated to be the two highest abundance genomes based on average coverage of the binned contigs.[57] These two organisms totaled to respective 52% relative abundance in the SDZ enrichment community (Figure 3a), highly enriched from the seed sludge, in which the relative abundance for *Pimelobacter* bin_SDZ1 and *Arthrobacter* bin_SDZ2 were only 0% and 0.02%. The organism *Pimelobacter* bin_SDZ1 had the type strain *Pimelobacter* simplex ATCC 6946[58] as the closest relative. Notably, the genome tree places *Arthrobacter* bin_SDZ2 genome in the same genus-level lineage with our previously isolated SDZ-degrading *Arthrobacter* sp. D2. *Arthrobacter* bin_SDZ2 had 100% average amino acid (AAI) and nucleotide acid identities (ANI),[59] to the strain *Arthrobacter* sp. D2, indicating they were the same strain. Also included in SDZ-degrading communities were low abundance (<6%) members of the genera *Frateuria*, *Achromobacter*, *Nitrobacter*, *Fimbriimonas*, and *Truepera*, along with members of the families *Comamonadaceae*, *Cytophagaceae*, *Chitinophagaceae*, *Bradyrhizobiaceae*, and *Micrococcaceae*. Presumably, community compositions obtained after long-term enrichment were expected to be optimal for the degradation of SDZ and its byproducts, and the deterministic influences of *Arthrobacter* sp. D2 and *Pimelobacter* bin_SDZ1 on the SDZ-degrading community profiles underscore their potential dominant roles for sulfonamide biodegradation processes.

2-AP disturbance induced clear changes in the community composition of SDZ enrichment. As determined by SIMPER analysis, abundance changes of *Arthrobacter* sp. D2, *Comamonadaceae* bin_SDZ3 and *Flavobacteriales* bin_SDZ9 genomes were the major drivers contributing to the compositional variation between the SDZ and 2-AP enrichment communities (Table S7). In comparison with the consortia using SDZ as the sole carbon source, the community using 2-AP as the sole carbon source was marked by substantial declines in relative abundances of *Arthrobacter* sp. D2, (from ∼25% to non-detectable level), and *Comamonadaceae* bin_SDZ3 (from 6% to 0.2%) (Figure 3b), indicating they might be responsible for initial SDZ breakdown. On the other hand, 2-AP promoted a large increase in the relative abundance of *Flavobacteriales* bin_SDZ9 (from 1% to 11%) which became the second most abundant genome in the 2-AP enrichment. At the same time, 2-AP enrichment community retained the notable dominance of *Pimelobacter* bin_SDZ1 (∼24%) as compared with the SDZ enrichment community (∼27%). Niche-based selection caused by resource availability is one of the determining factors for microbial community structure.[60,61] The evident community divergences between SDZ and 2-AP enrichments supported the niche differentiation hypothesis, in that only 2-AP-degrading specialist bacteria represented by *Pimelobacter* bin SDZ1 and *Flavobacteriales* bin SDZ9 were selected strongly by 2-AP supplementation.

Role of *Pimelobacter* sp. LG209 in SDZ degradation. As evidenced by the predominance of *Pimelobacter* bin_SDZ1 and *Arthrobacter* sp. D2 in the long-running SDZ enrichment communities, both microorganisms were presumed to have important roles in the aerobic SDZ degradation. Nevertheless, whether the microbial interactions between these two key bacteria are cooperative or competitive in SDZ biodegradation processes still remain elusive. Metagenomic investigations of the 2-AP enrichment community have shown the remarkable propensity of the *Pimelobacter* populations to use 2-AP as the sole carbon source. To isolate the *Pimelobacter* populations, serial dilutions of 2-AP enrichment culture were plated on MSM agar plate containing 2-AP as the sole carbon source at 30 °C which is the cultivation temperature used for the closest relatives of the *Pimelobacter* bin_SDZ1 (Suzuki et al., 1983). Individual colonies of 2-AP degraders on the plates were picked and further purified. Sanger sequencing of 16S rRNA gene of isolated colonies corroborated the purity of a *Pimelobacter* bacterium. The isolate, designated strain LG209, possessed exactly identical genomic sequences (100% AAI and ANI) to the population bin genome *Pimelobacter* bin_SDZ1 that was dominant in both SDZ and 2-AP enrichment cultures, suggesting the evident dominance of strain LG209 in both SDZ and 2-AP enrichment cultures.

To investigate the degradation possibility of SDZ or 2-AP by the strain LG209, this isolate was incubated with SDZ or 2-AP in three contrasting nutrient regimes at room temperature which was the same as the temperature for enrichment cultures. The results demonstrated that the strain LG209 lacked the SDZ degradation ability (Figure S4) while, contrastingly, exhibited high 2-AP degradation ability. During the incubation of LG209 with defined MSM supplemented with 200 mg/L 2-AP as the sole carbon source, the concentration of 2-AP decreased rapidly with increasing concentration of 4-OH-2-AP which was generated from the hydroxylation at the C4 position of the pyrimidine ring of 2-AP, and substantial TOC removal (∼81.7%) were observed at the end of the experiments (Figure S5). So far, the only known another 2-AP-degrading microorganism was *Terrabacter*-like bacterium isolated from soil that was treated with SDZ-containing manure for 3 y.[62]

SDZ Degradation by Coculture of D2 and LG209. The metagenomic analyses in this study provided insights into the vital roles of two populations, i.e. *Arthrobacter* sp. D2 and *Pimelobacter* sp. LG209, and how they may partner with each other in the SDZ degradation. To test the developed hypotheses about the partnership of D2 and LG209, the SDZ degradation by strain D2 was conducted at the same condition as the enrichment. As expected, strain D2 demonstrated the ability to degrade SDZ to 2-AP, generating 0.89 mol of 2-AP from 1 mol of the SDZ mother compound, i.e. 90% conversion from SDZ to 2-AP by pure culture of strain D2. However, SDZ mineralization by strain D2 was rather limited under that condition. Only around 23.6% TOC removal was observed. The pure culture of strain LG209 removed 81.7% TOC using 2-AP as the sole carbon source.

The SDZ degradation by the cocultures of strain D2 and LG209 was also examined under the same condition. In the

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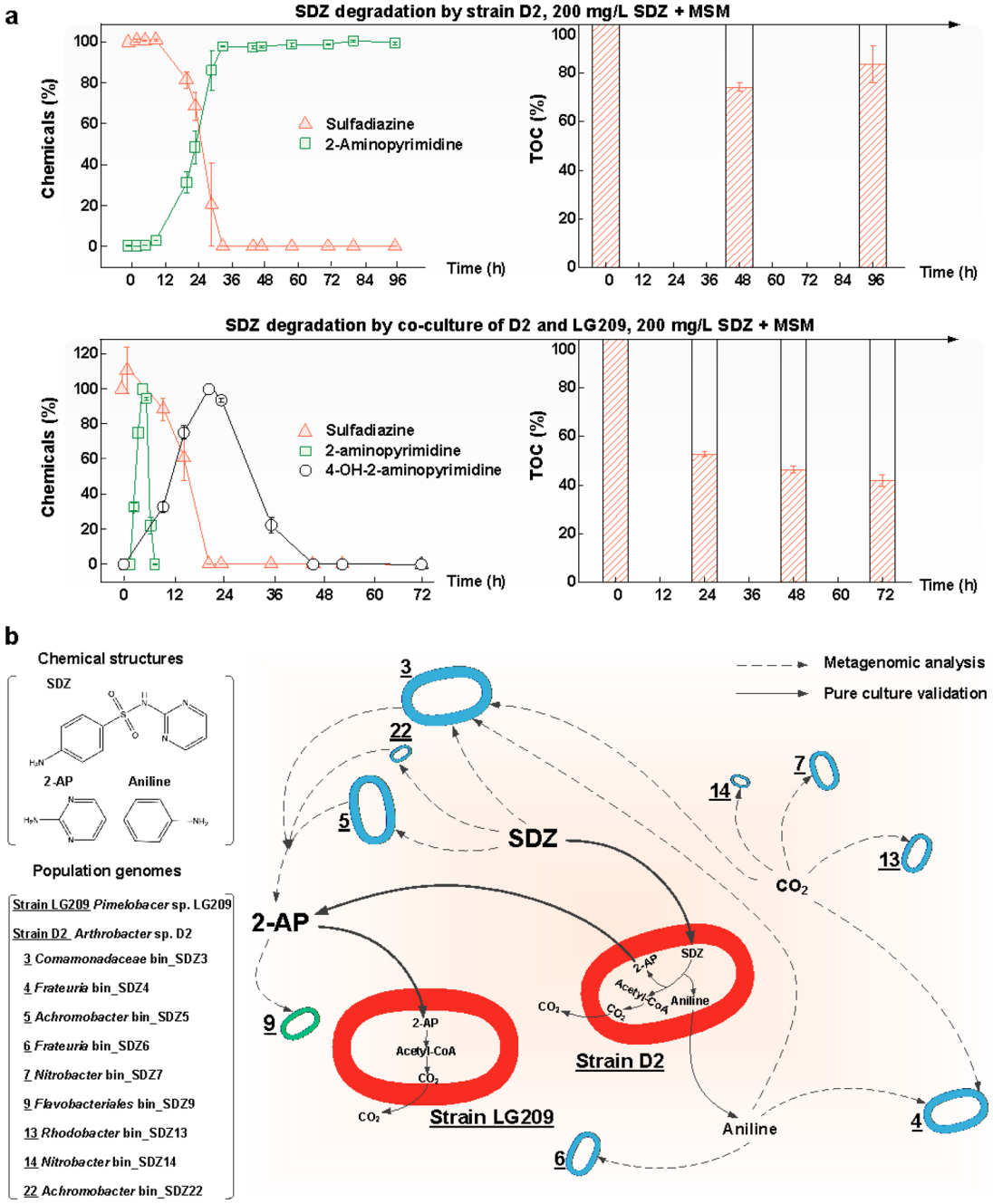

Figure 4. (a) Degradation and mineralization of SDZ by strain D2 alone (OD_{595} for D2 was 0.31 ± 0.02) and the coculture of strain D2 and LG209 (OD_{595} for D2 and LG209 were 0.32 ± 0.01 and 0.29 ± 0.01, respectively). (b) Schematic diagram illustrating community membership based on carbon flow in the SDZ enrichment. Symbol size is proportional to the genome relative abundance in the community. The color represents phylum-level taxonomy. Strain D2 was largely responsible for the initial breakdown of SDZ, producing 2-AP as the predominant metabolites and a small amount of aniline, and strain LG209 further mineralized 2-AP. 54 ± 1.3% TOC from SDZ was removed by these two strains, while other less abundant members participate in the SDZ mineralization by consuming catabolic byproducts presumably derived from primary degraders.

coculture using SDZ as the sole carbon source, SDZ decreased with quick buildup of 2-AP before the generation of 4-OH-2-AP, indicating the degradation process from SDZ to 2-AP and then to 4-OH-2-AP. Following the stoichiometry of SDZ degradation to 2-AP by strain D2 and 2-AP degradation by strain LG209, and considering the TOC of 2-AP is 40% of SDZ, it was estimated that the TOC removal by the coculture of these two strains was ~53% (= 23.6% (by D2) + 40% × 90% × 81.7% (by LG209)), which was highly consistent with the measured real TOC removal of 54 ± 1.3% in the coculture experiment (Figure 4a). The TOC removal percentage by these two populations was also highly consistent with their relative abundance in the SDZ enrichment culture (~25% for strain D2 and ~27% for strain LG209) (Tables S8 and S9).

Community Membership in SDZ Enrichment Culture. As demonstrated and discussed above, strain D2 was largely responsible for the initial breakdown of SDZ, generating 2-AP as the major intermediate products and a small amount of aniline, and strain LG209 further mineralized 2-AP. The coculture experiment showed that these two most prominent

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members in the SDZ enrichment consortia removed 54 ± 1.3% TOC, while the enrichment consortia could remove ~92% in total. Thus, there was ~38% TOC being consumed by other less abundant members in the SDZ enrichment community. Figure 4b illustrated the proposed community membership within the SDZ enrichment based on the experiment results in this study and analyses of the draft genomes of these populations. *Comamonadaceae* bin_SDZ3 was one of the three organisms having decreased relative abundance in the 2-AP enrichment compared to the SDZ enrichment community, showing changing patterns similar to that of strain D2. Thus, *Comamonadaceae* bin_SDZ3 might also participate in the initial step of SDZ degradation as one of the primary SDZ degraders, or alternatively, it may contribute to the aniline degradation.[63,64] Other proposed primary SDZ degraders were members of genus *Achromobacter*, which had demonstrated its sulfonamide-degrading ability by several *Achromobacter* pure cultures,[31,65] and degraded the sulfonamide along with accumulation of the heterocyclic moieties.[29] Two *Achromobacter* genomes (*Achromobacter* bin_SDZ5 and *Achromobacter* bin_SDZ_22) were found in low abundance in the SDZ enrichment community, indicating their less contribution in the total SDZ removal compared with other primary SDZ degraders in this study. Several bacteria may participate in SDZ degradation as the secondary degraders utilizing the degradation byproducts generated by primary SDZ degraders. As a bacterial population selected by 2-AP supplementations, *Flavobacteriales* bin_SDZ9 might be involved in degrading 2-AP produced by the primary SDZ degraders. Given the aniline-degrading ability being well identified in *Frateuria* pure cultures,[66,67] it was proposed that two *Frateuria* bacteria could degrade aniline in the SDZ-degrading enrichment community. Furthermore, except for degraders for SDZ and its byproducts, we also identified a few members (*Nitrobacter* bin_SDZ7, *Rhodobacter* bin_SDZ13 and *Nitrobacter* bin_SDZ 14) capable of aerobic carbon fixation (Figure S6).

Environmental Relevance. Using microcosm-scale enrichment communities, here we provided the first attempt to reveal roles of major microorganisms present in SDZ biodegradation. By incorporating genome-resolved metagenomics and pure culture validations assays, this study illuminated the fundamentally distinguished roles of individual isolate *Pimelobacter* sp. LG209 and *Arthrobacter* sp. D2 in initiating SDZ biodegradation. Their cooperative degradation process, in which SDZ was initially degraded by strain D2 with significant generation of 2-AP for strain LG209, was assumed to be the most prominent degradation pattern within the long-running sulfadiazine enrichment. This study demonstrated that metagenomic investigations of artificially enriched microbial communities enabled complementation of information derived from cultivated isolate representatives. Mechanistic understanding on relationship between the sulfonamide-degrading specialist and other community members that constitute part of an ecological network will help the empirical development of sulfonamide bioremediation. However, the growth and activity of sulfonamide degraders *in situ* have still been confronted by impacts caused by unknown microbial diversity of naturally occurring microbiomes, and by different nutritional substrates and physiological conditions in the open and complex environments. Integration of metagenomic analyses with cultivation-based techniques in future inquiries should allow complementation and convergence of information to address the above issues, which could further pave the way for more accurate extrapolation of laboratory-scale experiment results to the natural environments. Overall, identification of functionally significant bacteria and their interactions with other community members during sulfonamide biodegradation are crucial for designing successful remediation schemes. Nevertheless, since the distribution of sulfonamide-resistant degraders in the open environment may contribute to the elevated levels of antibiotic resistance genes which are increasingly treated as emerging contaminants, when developing bioremediation strategies using these degraders, it is of great importance to acknowledge the potential risks carried by certain degraders, and perform appropriate control methods like using enclosed systems coupled with postcontrol strategies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.7b05913.

Nine tables (Tables S1−S9) and six figures (Figures S1−S6) as mentioned in the text (PDF)

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Notes

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