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Biodegradation of Sulfamethoxazole in Milkfish (*Chanos chanos*) Pond Sediments

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Abstract: To cope with bacterial infections, broad-spectrum antibiotics such as sulfonamides have been largely used for intensive coastal aquaculture. Sulfonamides are stable and difficult to remove by conventional wastewater treatment. Environmental pollution will occur if sulfonamide-containing aquaculture wastewater is discharged into rivers and oceans. In this study, high salinity-tolerant bacterial strains A12 and L with sulfamethoxazole (SMX)-degrading ability from milkfish (*Chanos chanos*) culture pond sediments with SMX were isolated, identified, and characterized. The degradation of SMX and the changes in the bacterial community in milkfish culture pond sediments were assessed. Phylogenetic analysis using 16S rRNA gene sequences suggested that bacterial strain A12 was very close (99% sequence identity) to *Vibrio* sp., and bacterial strain L was very close (99% sequence identity) to *Pseudomonas* sp. Aerobic and anaerobic batch and continuous SMX addition experiments indicated that bacterial strains A12 and L could enhance SMX degradation in milkfish culture pond sediments. Different microbial community compositions under aerobic and anaerobic conditions exhibited different SMX-degrading abilities. The results of this study suggest that bacterial strains A12 and L provide a solution for treatment of wastewater and sediment from SMX-contaminated high salinity milkfish culture ponds.

Keywords: sulfamethoxazole; milkfish pond sediment; bioremediation

1. Introduction

Intensive farming is usually used for aquaculture. Due to efforts to increase productivity, aquaculture systems are suffering from overcrowding and disease outbreaks. Most infectious diseases in aquaculture are bacterial [1]. Antibiotics such as sulfonamides were usually used in aquaculture to prevent and treat bacterial infectious diseases [2]. As a consequently, residues of antibiotics remain in fishery products, and concerns have been raised because dietary consumption is the main way that humans are exposed to organic pollutants [3,4]. Moreover, antibiotics such as sulfonamides are difficult to remove through conventional wastewater treatment. Environmental pollution will occur if sulfonamide-containing wastewater is discharged into rivers and oceans [5,6].

Coastal aquaculture is a vast industry because of the large demand for marine products. Milkfish (*Chanos chanos*) is an important coastally cultured fish among the south Asian countries [7]. Milkfish are euryhaline fish that are usually cultured in a mixture of fresh water and sea water [8]. To prevent environmental pollution, wastewater after fish breeding must be properly treated. To perform biodegradation/bioremediation of the residual sulfonamide antibiotics in the water environment, sulfonamide-degrading bacteria that are tolerant of high salinity are necessary. Moreover, little is

known about the degradation of sulfonamide and the change in the bacterial community of milkfish culture pond sediments.

The aim of this study was the isolation, identification, and characterization of sulfamethoxazole (SMX)-degrading bacteria from milkfish culture pond sediments. Batch and SMX continuous addition experiments were performed to assess SMX degradation with degrading bacteria in milkfish culture pond sediments. The effects of these bacteria on bacterial community composition in the sediment of milkfish culture ponds were revealed.

2. Materials and Methods

2.1. Chemicals

Chemicals and target compounds, SMX, sulfadimethoxine (SDM), and sulfamethazine (SMZ) (99.0% purity) used in this work were purchased from Sigma-Aldrich (St. Louis, MO, USA). Marine broth and agar were purchased from BD Difco™ (Franklin Lakes, NJ, USA).

2.2. Sediment Sampling and Sampling Site

In July 2016, sediment samples were collected from a farm-scale milkfish pond at the Mariculture Research Center, Fisheries Research Institute, Tainan, Taiwan. The latitude and longitude of the sampling site is 23°07′20.3″ N 120°04′47.8″ E. Samples were collected randomly, in triplicate, from an area of about 1 m^2. The physicochemical parameters of the mixture of milkfish pond sediment samples were pH (8.5 ± 0.3), salinity (32 ± 2 ‰), total nitrogen (811 mg kg^{-1}), total phosphate (196 mg kg^{-1}), organic matter (0.97%), sand (59.4%), silt (25.1%), and clay (15.5%). The three target sulfonamides, SMX, SMZ, and SDM were not detected in the sediment samples of milkfish ponds. The processes of adaptation was conducted by adding 2 mg L^{-1} to 500 g of sediment and incubation under 25 °C in the dark for 3 months.

2.3. Experimental Setting

In this study, four sets of experiments were conducted. The first set of experiments was implemented to test the sulfonamide-degrading ability of milkfish pond sediments. For each experiment, 125-mL bottles with 45 mL of medium and 5 g of milkfish pond sediments were used. The sulfonamide concentrations used in the experiments were 2 and 20 mg L^{-1} each of SMX, SDM, and SMZ. Samples for sterile controls were autoclaved at 121 °C for 30 min [9]. Aerobic experiments were incubated with shaking at 25 °C in the dark. Anaerobic experiments were performed in an anaerobic glove box (Forma Scientific, model 1025 S/N, USA) filled with N_2 (85%), H_2 (10%), and CO_2 (5%) gases. Bottles were capped with rubber stoppers and incubated static at 25 °C in the dark. Each treatment of the experiments was repeated in triplicate. Samples were collected to check residual sulfonamides by HPLC. Bacterial communities were analyzed by next-generation sequencing (NGS).

The second set of experiments was designed to isolate and identify SMX-degrading bacteria and test SMX degradation abilities. Marine agar plates containing 2 mg L^{-1} SMX under aerobic and anaerobic conditions were used to isolate SMX-degrading bacteria. Individual colonies were collected, and DNA were amplified by PCR with the F8 and R1510 primers. The 16S rRNA gene amplicons were sequenced by the ABI Prism automatic sequencer, and the database was searched using the BLAST of the National Center for Biotechnology. Phylogenetic analysis was performed using MEGA 7 [10]. To confirm the ability of SMX degradation, experiments were performed using the isolated bacterial strains (10^5 CFU mL^{-1}), 50 mL of medium, and 2 mg L^{-1} SMX under aerobic and anaerobic conditions. Samples were collected to analyze residual SMX by HPLC.

The goal of the third and fourth sets of experiments was to test the aerobic and anaerobic SMX degradation efficiency in milkfish pond sediments. For the third set of experiments, aerobic and anaerobic batch experiments each containing an SMX-degrading bacteria strain (10^5 CFU mL^{-1}), 5 g of sediment, 45 mL of medium, and 2 mg L^{-1} of SMX were conducted. Samples were collected to

analyze residual SMX. The fourth set of experiments were conducted using 1000-mL bottles with 450 mL of medium and 50 g of sediment, with or without degrading bacteria (10^5 CFU mL^{-1}) under aerobic and anaerobic conditions. Then, 2 mg L^{-1} of SMX was used for the first and second additions, and 10 mg L^{-1} of SMX was used for the third and fourth additions. SMX was re-added, while SMX decreased to ND (not detected). The process was repeated by adding SMX four times. The experiments lasted for 80 days. Samples were collected to survey residual SMX and to analyze the bacterial community by NGS.

2.4. Analysis of Sulfonamides

A concentration of 10,000 mg/L sulfonamide antibiotic stock solution was prepared by weighting 0.5 g of a sulfonamide antibiotic and dissolving it in a solvent of methanol: acetonitrile = 1:1 in a dosage bottle to 50 mL. The standards of 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50 and 100 mg L^{-1} were obtained by serial dilution with a solvent of methanol: acetonitrile = 1:1 and analyzed by HPLC. Calibration curves of the three sulfonamide antibiotics were computed by linear regression. The detection limits for SMX, SDM, and SMZ were 0.1 mg L^{-1}. The 2, 10, and 20 mg L^{-1} working solutions used in experiments were obtained by dilution of the stock solution using a solvent of methanol: acetonitrile = 1:1. Sulfonamides were extracted and analyzed as previously described [11]. Briefly, the 0.5-mL aqueous phase samples were collected, and equal volumes of extraction solution (containing water with 0.1% formic acid: acetonitrile: methanol = 10:3:1) were added, mixed, and centrifuged at 10,000 rpm for 5 min. The supernatant was filtered with a 0.22-mm filter into a vial. Samples were extracted twice, and the products were pooled for HPLC analysis. Sulfonamides were analyzed using an Agilent 1260 HPLC system equipped with a 4.6 × 250-mm column (Zorbax Eclipse Plus C18, Agilent) and a photodiode array detector monitoring at 270 nm. The remaining percentage R_p is computed by the formula: $R_p = (RC_{SA}/IC_{SA}) \times 100\%$, where RC_{SA} is the residue sulfonamide concentration. IC_{SA} is the initial sulfonamide concentration. The sulfonamide degradation rate constants (k) and half-lives ($t_{1/2}$) were estimated by a linear regression equation (a first-order decay model): $t = -\ln(C/C_0)/k$, where C_0 is the initial concentration, C is the substrate concentration, t is the time period, $t_{1/2}$ is the half-life, and k is the degradation rate constant [12].

2.5. Scanning Electron Microscope

Bacterial samples were fixed in 2.5% glutaraldehyde for 2 h at 4 °C and post-fixed in 1% osmium tetroxide in the same buffer for 1 h. Fixed samples were dehydrated in a graded ethyl alcohol series, critical point-dried, and gold coated. A Hitachi S-4700 scanning electron microscope (USA) was used to take photographs.

2.6. Next-Generation Sequencing and Data Analysis

NGS data analysis was performed as described previously [11]. Briefly, the DNA samples were isolated by the PowerSoil DNA Isolation Kit (QIAGEN). The V5–V8 regions of 16S rRNA gene were amplified using the 5′ primer containing an Illumina adaptor (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3′) and a 16S rRNA gene specific sequence (5′-CCTACGGGNBGCASCAG-3′). The sequence of the 3′ primer contained an Illumina adaptor (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3′) and a 16S rRNA gene specific sequence (5′-GACTACNVGGGTATCTAATCC-3′). NGS was performed at the Genome Center of National Yang-Ming University, Taiwan using the MiSeq platform (Illumina, Inc., San Diego, CA, USA). Sequences that passed the chimera check were applied to the classifier software of the Ribosomal Database Project (http://pyro.cme.msu.edu/). Similarity (95%) was used for the assignment of taxonomic classification. The differences of proportions between samples were identified by the Mann–Whitney U-Test by R (https://www.r-project.org/). P values of less than 0.05 were considered statistically significant. Heatmap and cluster analysis were computed and plotted using the R package pheatmap.

3. Results and Discussion

3.1. Test of Sulfonamide Degradation in Milkfish Pond Sediments

The milkfish pond sediments were used to test the biodegradation of the three sulfonamides. Among the three sulfonamides, only SMX could be efficiently degraded. As shown in Figure 1, only SMX was degraded in the milkfish pond sediments under aerobic and anaerobic conditions at the end of a 40-day incubation period.

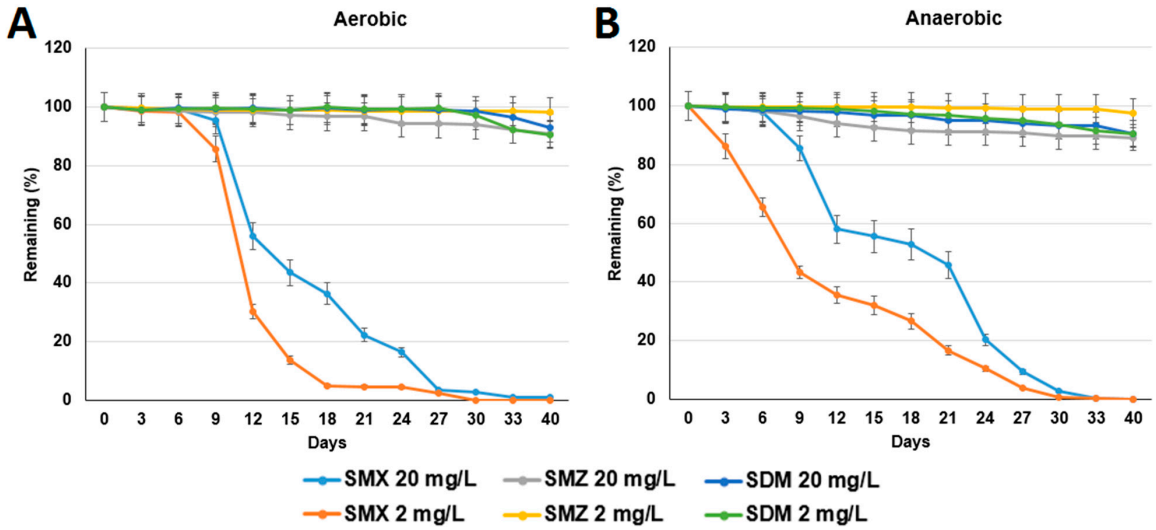

Figure 1. Biodegradation of three sulfonamides in milkfish (*Chanos chanos*) pond sediments under aerobic (**A**) and anaerobic (**B**) conditions. SMX: sulfamethoxazole, SDM: sulfadimethoxine, SMZ: sulfamethazine. Data from three independent experiments are presented as the mean ± SE.

The rate constants (k) and half-lives ($t_{1/2}$) of the three sulfonamides in the experiments shown in Figure 1 were estimated and listed in Tables S1 and S2. The remaining percentages of the three sulfonamides in the sterilized milkfish pond sediments under aerobic and anaerobic conditions were ranged from 97.8% to 99.2% at the end of incubation period of 40 days. These results reveal that aerobic and anaerobic SMX degradation were due to microbial actions. The interaction between sulfonamides and microbes may have been affected by the electronegative effects of functional groups of sulfonamides that may have hindered degradation [13]. Therefore, SMX is easier to be degraded than SMZ and SDM in milkfish pond sediments. A concentration of 2 mg L^{-1} SMX was used for the following experiments.

3.2. Isolation and Identification of Sulfamethoxazole-Degrading Bacteria

To isolate SMX-degrading bacteria for further application, SMX was added to the sediment of milkfish ponds and incubated under aerobic or anaerobic conditions for three months. Aerobic and anaerobic microbial communities before and after SMX adaptation are shown in Figures 2 and 3, respectively. For SMX adaptation under aerobic conditions, Bacteroidetes increased from 10% to 30% (Figure 2A). Only one reported SMX-degrading bacterial genus (*Hyphomicrobium*) was found before SMX adaptation [14]. Four reported SMX-degrading bacterial genera (*Brevundimonas*, *Escherichia/Shigella*, *Hyphomicrobium*, and *Shewanella*) and one isolated in this study (*Vibrio*) were found after SMX adaptation [14–17] (Figure 2B). For SMX adaptation under anaerobic conditions, archaea (Euryarchaeota, Pacearchaeota) decreased from 39% to 4% (Figure 3A). Proteobacteria increased from 38% to 56%. One reported SMX-degrading bacterial genus (*Escherichia/Shigella*) [16] and one isolated in this study (*Marinobacter*) were found before SMX adaptation. Three reported SMX-degrading bacterial genera (*Escherichia/Shigella*, *Flavobacterium*, and *Pseudomonas*) [15,16,18] and one isolated in this study (*Marinobacter*) were found after SMX adaptation (Figure 3B). More reported SMX-degrading bacterial

genera were observed after SMX adaptation under both aerobic and anaerobic conditions, indicating that SMX-degrading bacteria enrichment was achieved.

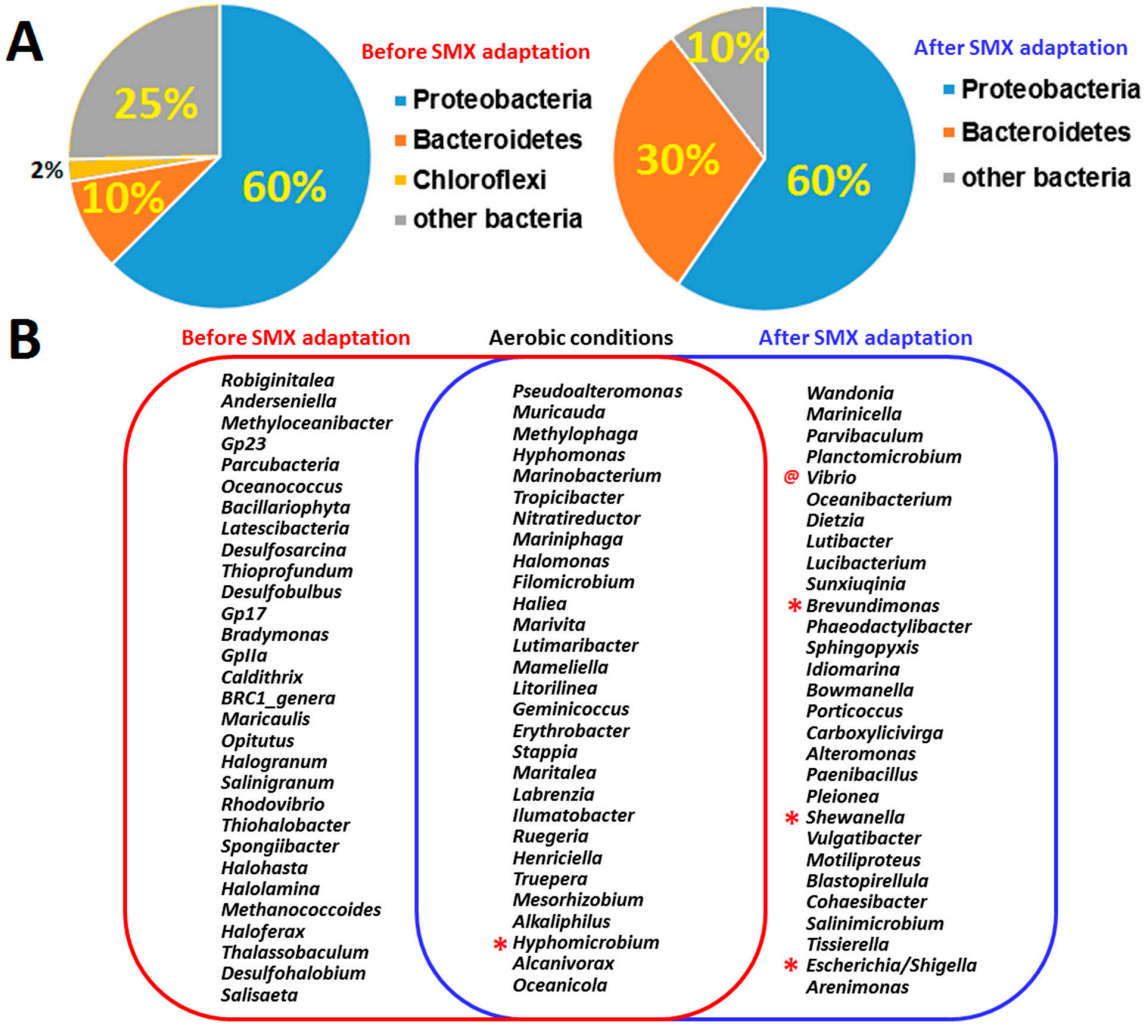

Figure 2. Bacterial/archaeal community in milkfish (*Chanos chanos*) pond sediments under aerobic condition before and after sulfamethoxazole (SMX) adaptation. (**A**) Major bacterial/archaeal community (phylum level) in the sediments under aerobic conditions before and after SMX adaptation. (**B**) Major bacterial/archaeal community (genus level) in the sediments under aerobic conditions before and after SMX adaptation. Red "@"s indicate SMX-degrading bacteria that were isolated in this study.

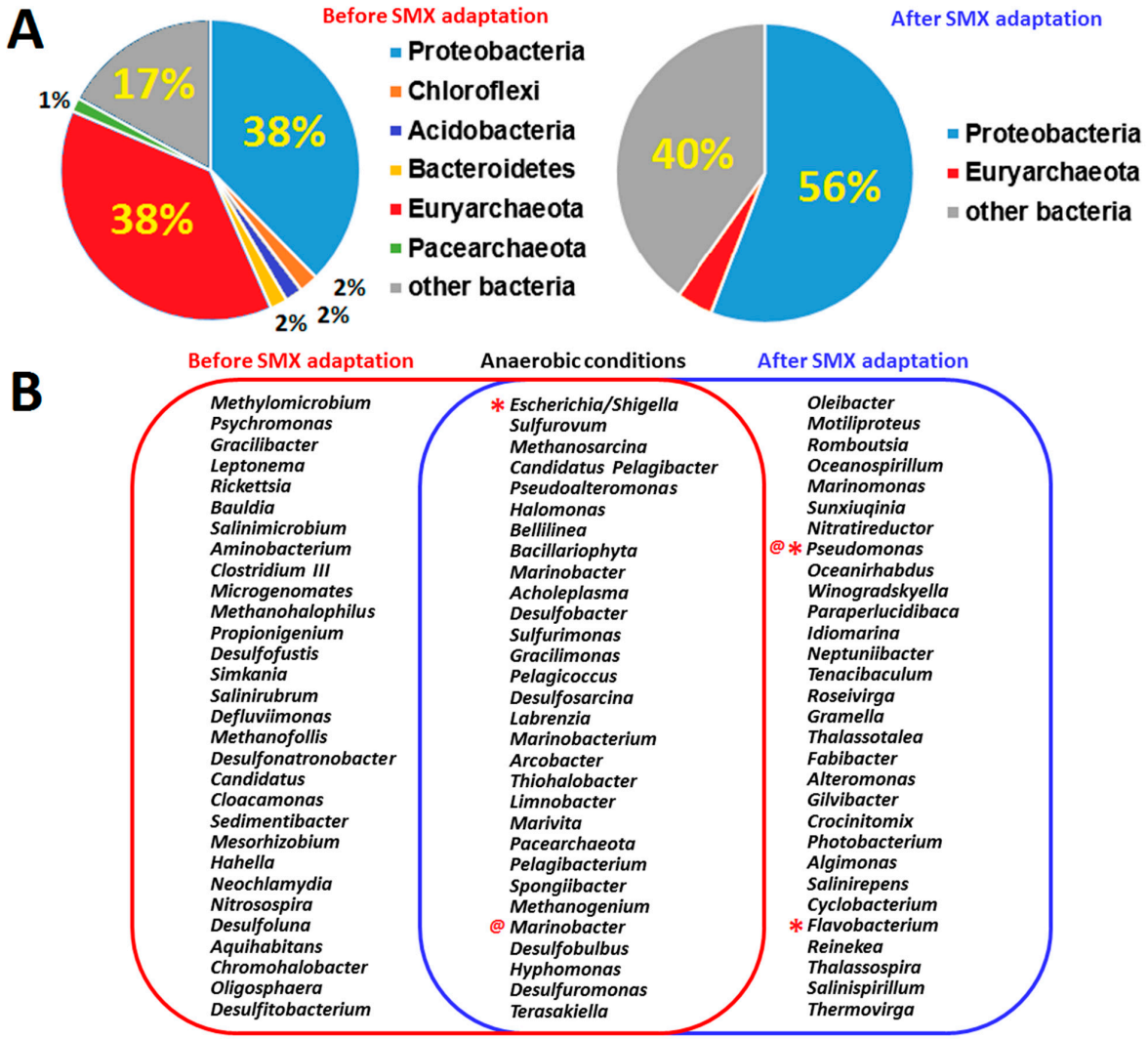

Figure 3. Bacterial/archaeal community in milkfish (*Chanos chanos*) pond sediments under anaerobic condition before and after sulfamethoxazole (SMX) adaptation. (**A**) Major bacterial/archaeal community (phylum level) in the sediments under anaerobic conditions before and after SMX adaptation. (**B**) Major bacterial/archaeal community (genus level) in the sediments under anaerobic conditions before and after SMX adaptation. Red stars indicate SMX-degrading bacteria that have been reported. Red "@"s indicate SMX-degrading bacteria that were isolated in this study.

The isolation of SMX degrading bacteria was performed using marine agar plates containing 2 mg L^{-1} SMX. Six bacterial strains (A1, A2, A12, A15, A16, and A20) and two bacterial strains (L and M) under aerobic and anaerobic conditions, respectively, were found to have the ability to use SMX as a carbon source and degrade SMX. SMX-degrading ability was tested under aerobic and anaerobic conditions. As shown in Figure 4A, SMX (2 mg L^{-1}) was completely degraded by the bacterial strains A2 and A12 under aerobic conditions within 10 days. Under anaerobic conditions, SMX (2 mg L^{-1}) was completely degraded by the bacterial strains L and M within 24 days (Figure 4B). The rate constants (k) and half-lives ($t_{1/2}$) of the SMX in the experiments shown in Figure 4 were estimated and listed in Table S3.

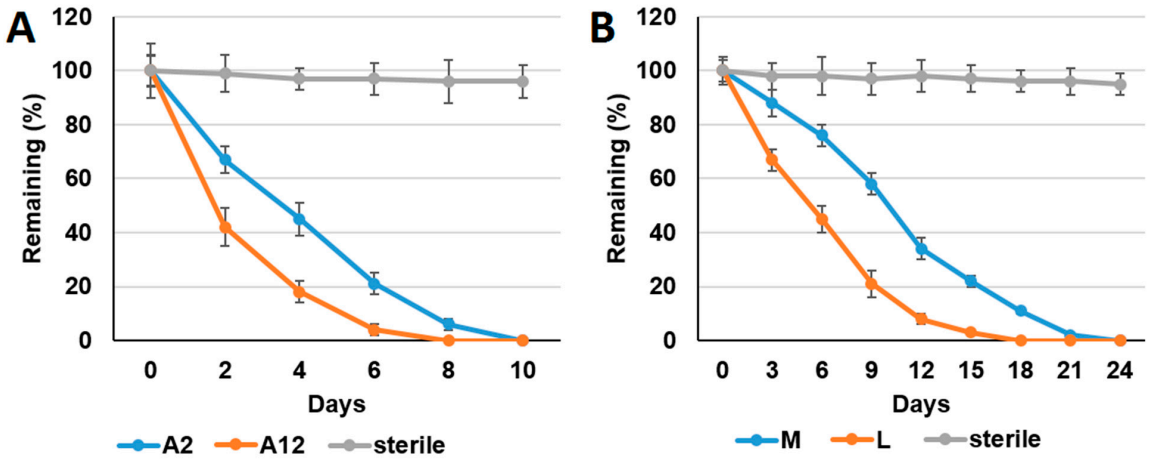

Figure 4. Biodegradation of sulfamethoxazole (SMX) by the four isolated bacterial strains (A2, A12, L, and M) without fish pond sediments. (**A**) Aerobic SMX biodegradation by strains A2 and A12. (**B**) Anaerobic SMX biodegradation by strains L and M. Data from three independent experiments are presented as the mean ± SE.

Phylogenetic analysis using 16S rRNA sequences indicated that bacterial strains A2 and A12 are very close (99% sequence identity) to *Vibrio* sp., and bacterial strains L and M are very close (99% sequence identity) to *Pseudomonas* sp. and *Marinobacter* sp., respectively (Figure 5 and Table S4). The morphology of the four bacterial strains (curved rod shape for *Vibrio* sp. and rod shape for *Pseudomonas* sp. and *Marinobacter* sp.) confirmed by scanning electron microscopy is shown in Figure 6.

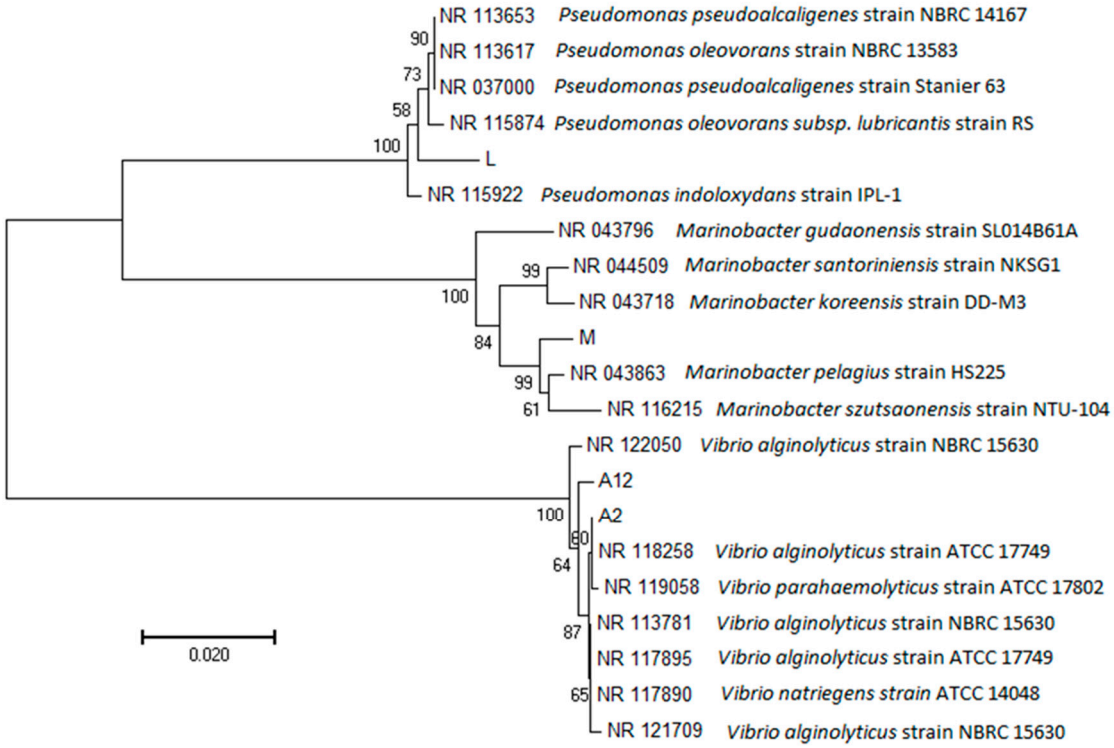

Figure 5. Phylogenetic analysis of 16S rRNA sequences of the four bacterial strains (A2, A12, L, and M). The ruler indicates the scale of the branch length of the tree, which estimates the average number of nucleotide substitutions per site. Bootstrapping values at each branch point indicate times out of 1000 the same branch was observed when repeating the phylogenetic reconstruction.

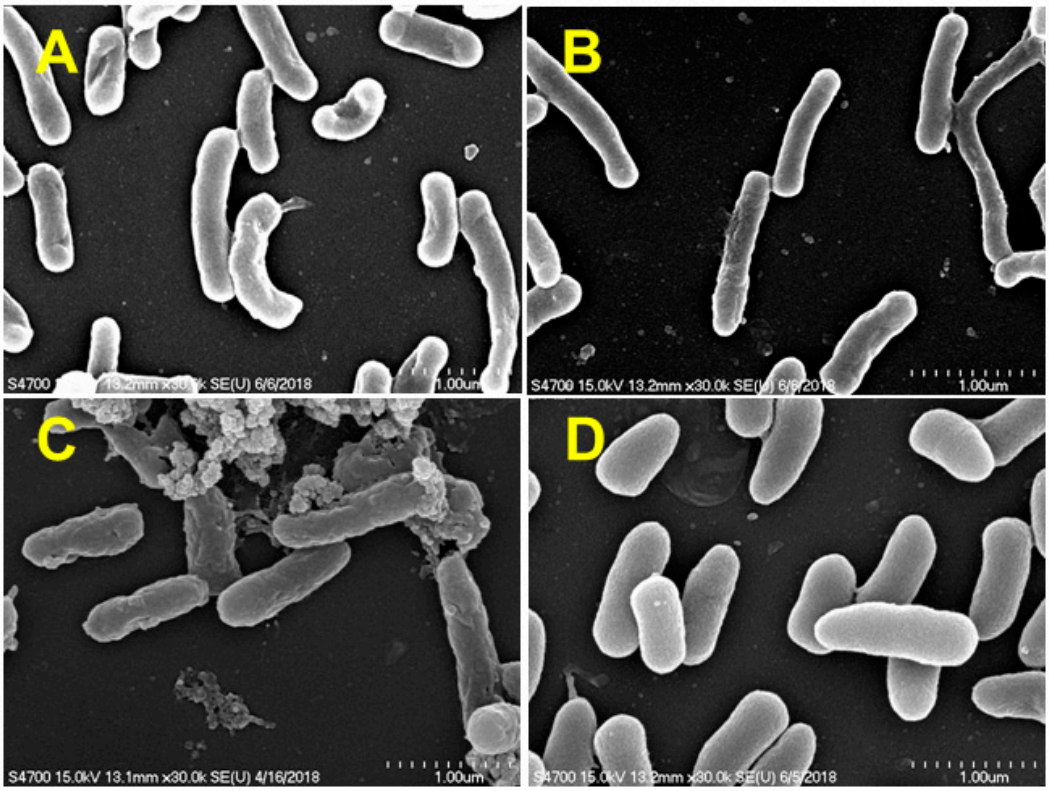

Figure 6. Photo pictures of isolated SMX-degrading bacteria by scan electron microscopy. (**A**) Bacteria strain A2. (**B**) Bacteria strain A12. (**C**) Bacteria strain L. (**D**) Bacteria strain M.

3.3. Test of Sulfamethoxazole-Degrading Ability of Isolated Bacteria

To test the SMX-degrading ability of the four bacterial strains (A2, A12, M, and L) in milkfish pond sediments, batch experiments were performed. In the sterilized controls, the SMX residues were all above 98% after 8 and 24 days of incubation under aerobic and anaerobic conditions. Therefore, the decomposition of SMX should be mainly due to microbial processes. The results indicated that SMX (2 mg L^{-1}) was completely degraded with bacterial strains A2 and A12 under aerobic conditions (Figure 7A), and completely degraded with bacterial strains L and M under anaerobic conditions (Figure 7B). The rate constants (k) and half-lives ($t_{1/2}$) of the SMX in the experiments shown in Figure 7 were estimated and listed in Table S5. These results indicate that the addition of isolated bacterial strains A2, A12, L, and M enhance SMX degradation in milkfish pond sediments. Based on the results of the SMX-degrading ability tests, bacterial strains A12 and L were used to perform aerobic and anaerobic continuous addition experiments, respectively.

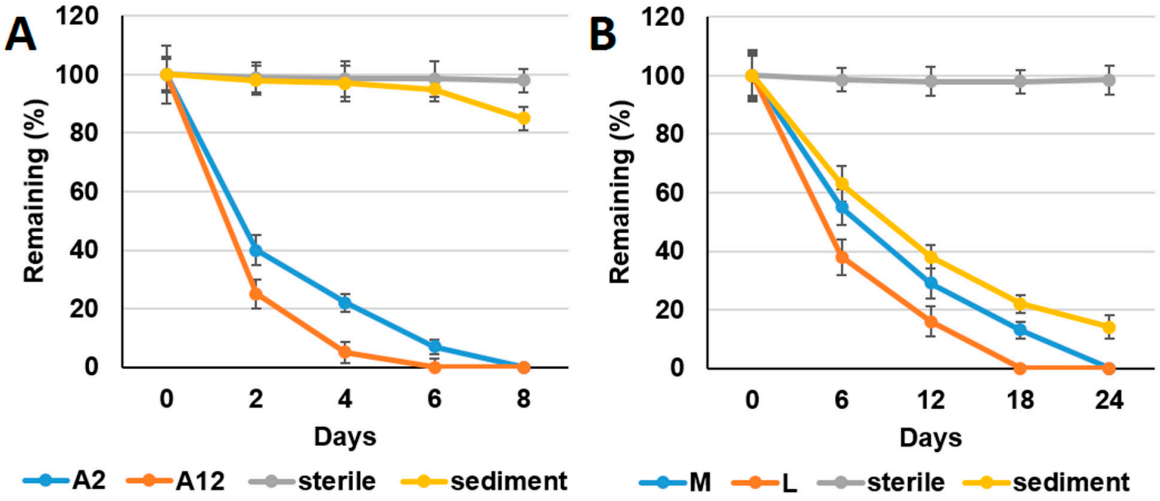

Figure 7. Biodegradation of sulfamethoxazole (SMX) by the four bacterial strains (A2, A12, L, and M) with fish pond sediments. (**A**) Aerobic SMX biodegradation by strains A2 and A12. (**B**) Anaerobic SMX biodegradation by strains L and M. Data from three independent experiments are presented as the mean ± SE.

SMX degradation in aerobic readdition experiments is shown in Figure 8A. The SMX in the readdition experiment with strain A12 was almost completely degraded on days 7, 21, 42, and 56. The SMX concentrations of the readdition experiment without strain A12 were 1.38, 2.61, 7.01, and 13.9 mg L^{-1} on days 7, 21, 42, and 56, respectively. The rate constants (k) and half-lives ($t_{1/2}$) of the SMX after the third and fourth additions shown in Figure 8A were estimated and listed in Table S6.

The results of SMX degradation in anaerobic readdition experiments are shown in Figure 8D. The SMX concentrations in the readdition experiment with strain L were 1.38, 1.71, 0.79, and 0 on days 7, 21, 42, and 81. The SMX concentrations in the readdition experiment without strain L were 1.75, 2.87, 10.88, and 8.01 mg L^{-1} on days 7, 21, 42, and 81, respectively. The rate constants (k) and half-lives ($t_{1/2}$) of the SMX after the third and fourth additions shown in Figure 8D were estimated and are listed in Table S7. The results indicate that the ability of bacterial strains A12 and L to enhance SMX degradation in milkfish pond sediments could be increased by the readdition of SMX under both aerobic and anaerobic conditions.

The degradation rates of the low SMX concentration (2 mg L^{-1} for the first and second additions) for both aerobic and anaerobic continuous addition experiments were similar to the degradation rates in the batch experiments (Figure 7A,B). In the high SMX concentration (10 mg L^{-1} for third and fourth additions) conditions, the degradation rate increased. It seems likely that SMX adaptation occurred in the readdition experiments, which led to SMX degradation at the late stages of the experiments without the addition of bacterial strains A12 and L. The degradation rate increased with SMX readdition (Tables S6 and S7). Microbes with SMX-degrading ability may increase in response to SMX readdition [11].

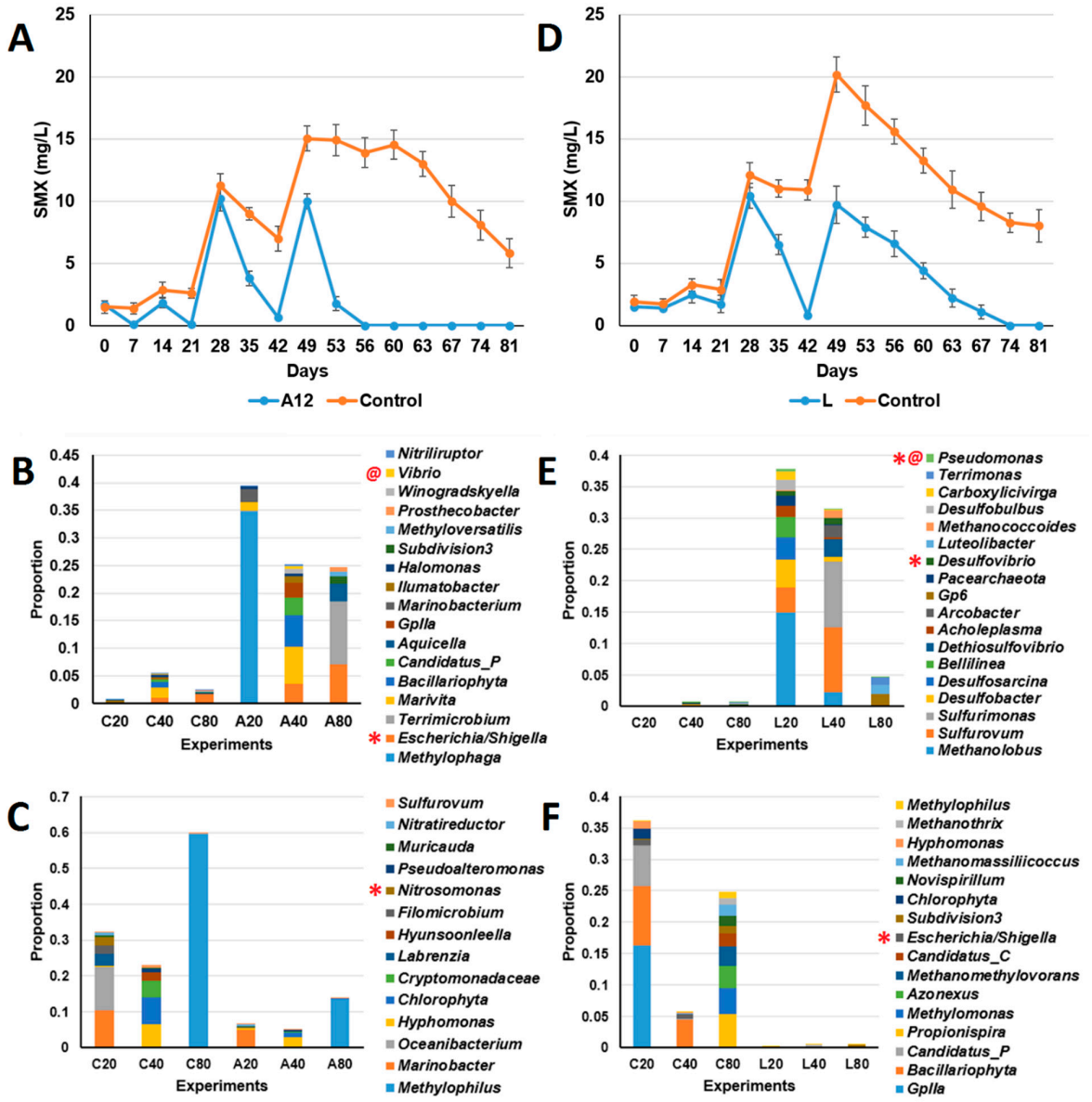

Figure 8. Biodegradation of sulfamethoxazole (SMX) by bacterial strains A12 and L (with fish pond sediments) in aerobic and anaerobic readdition experiments, respectively. (**A**) Aerobic SMX biodegradation with/without strain A12. (**B**) Major representatives of the microbial community in aerobic experiments with strain A12. (**C**) Major representatives of the microbial community in aerobic experiments without strain A12. (**D**) Anaerobic SMX biodegradation with/without strain L. (**E**) Major representatives of the microbial community in anaerobic experiments with strain L. (**F**) Major representatives of the microbial community in anaerobic experiments without strain L. Red stars indicate that SMX-degrading bacteria have been reported. Red "@"s indicate SMX-degrading bacteria isolated in this study. Proportions of microbes with a statistically significant difference between experiments with and without bacterial strains A12 and L were identified using the Mann–Whitney U-test ($p < 0.05$).

Aerobic and anaerobic microbial communities of continuous addition experiments are shown in Figure S1. Two major stages (before day 40 and after day 40) were found in the results of the cluster analysis. The microbial community compositions before day 40 were largely distinct from the microbial community compositions after day 40. These results indicated that the microbial communities shifted after 40 days of SMX readdition, which may be due to SMX adaptation.

Seventeen bacterial genera, including one reported SMX-degrading bacterial genus (*Escherichia*/*Shigella*) [16], exhibited a higher proportion in the experiment with strain A12 (Figure 8B). Fourteen bacterial genera, including one reported SMX-degrading bacterial genus (*Nitrosomonas*) [14], exhibited a higher proportion in experiments without strain A12 (Figure 8C). Eighteen bacterial genera, including two reported SMX-degrading bacterial genera (*Pseudomonas* and *Desulfovibrio*) [15,19], exhibited a higher proportion in experiments with strain L (Figure 8E). Fourteen bacterial genera, including one reported SMX-degrading bacterial genus (*Escherichia*/*Shigella*) [16], exhibited a higher proportion in experiments without strain L (Figure 8F). These results indicated that different microbial communities occurred after 40 days of SMX readdition under aerobic and anaerobic conditions. Moreover, different microbial community compositions exhibited different SMX-degrading abilities (Figure 8A,D).

Mariculture and coastal aquaculture activities can lead to pollution by micropollutants (e.g., antibiotics) in coastal seawater, which host the highest biodiversity in the world. Thus, it is important to investigate bioremediation solutions for organic micropollutants in coastal waters [20]. The study of Benotti and Brownawell (2009) indicated that the microbial degradation of pharmaceuticals, such as sulfamethoxazole and trimethoprim, in and near a wastewater-polluted estuarine and coastal seawater and sediments, is much slower than the degradation of biomolecules, such as glucose and amino acids [13]. It has been reported that chronic exposure to low concentrations and legal aquaculture doses of SMX causes systemic adverse effects and provoke differential human health risks in Nile tilapia [21]. For aquaculture, SMX is commonly administered at 100 to 200 mg kg^{-1} fish body weight/day for five days [22], depending on fish species, infection, and country-specific legal requirements. The SMX concentrations used in this study were extremely higher than what would be expected in the environment. However, they are reasonable for aquaculture wastewater treatment.

4. Conclusions

In this study, salinity-tolerant SMX-degrading bacteria A12 and L from milkfish pond sediments were isolated, identified, and characterized. SMX-degrading bacterial strains A2 and A12 are very close to *Vibrio* sp., and bacterial strains L and M are very close to *Pseudomonas* sp. and *Marinobacter* sp., respectively. Aerobic and anaerobic batch and readdition experiments indicated that bacterial strains A12 and L exhibited high performance in SMX degradation in milkfish pond sediments. The results of this study suggest that bacterial strains A12 and L provide a potential solution for the treatment of wastewater and sediment from high-salinity aquaculture ponds with SMX contamination. These bacteria may also be applicable for the bioremediation of SMX-contaminated mangrove, estuarine, and coastal sediments.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/9/19/4000/s1, Figure S1: Biodegradation of sulfamethoxazole (SMX) by bacterial strains A12 and L (with fish pond sediment) in aerobic and anaerobic readdition experiments, respectively, Table S1: Aerobic SA degradation rate constants (k) and half-lives ($t_{1/2}$) in Figure 1A, Table S2: Anaerobic SA degradation rate constants (k) and half-lives ($t_{1/2}$) in Figure 1B., Table S3: SMX degradation rate constants (k) and half-lives ($t_{1/2}$) in Figure 4, Table S4: 16S rRNA gene sequence analysed by the NCBI Blast in Figure 5, Table S5: SMX degradation rate constants (k) and half-lives ($t_{1/2}$) in Figure 7, Table S6: The aerobic SMX degradation rate constants (k) and half-lives ($t_{1/2}$) following the 3rd and 4th additions of SMX during 81 d of incubation in Figure 8A, Table S7: The anaerobic SMX degradation rate constants (k) and half-lives ($t_{1/2}$) following the 3rd and 4th additions of SMX during 81 d of incubation in Figure 8D.

Author Contributions: Investigation, B.-V.C., W.-L.C., D.L.K. and C.-W.Y.; Methodology, S.-L.Y. and D.L.K.; Project administration, W.-L.C.; Writing—original draft, B.-V.C. and C.-W.Y.; Writing—review and editing, B.-V.C. and C.-W.Y.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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