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Biodiversity of Microorganisms Colonizing the Surface of Polystyrene Samples Exposed to Different Aqueous Environments

Tatyana Tourova [1], Diyana Sokolova [1], Tamara Nazina [1,*], Denis Grouzdev [2], Eugeni Kurshev [3] and Anatoly Laptev [3]

[1] Winogradsky Institute of Microbiology, Research Center of Biotechnology, Russian Academy of Sciences, 119071 Moscow, Russia; tptour@rambler.ru (T.T.); sokolovadiyana@gmail.com (D.S.)
[2] Institute of Bioengineering, Research Center of Biotechnology of the Russian Academy of Sciences, 119071 Moscow, Russia; denisgrouzdev@gmail.com
[3] Federal State Unitary Enterprise "All-Russian Scientific Research Institute of Aviation Materials", State Research Center of the Russian Federation, 105005 Moscow, Russia; kurshev.viam@mail.ru (E.K.); laptevab@viam.ru (A.L.)
* Correspondence: nazina@inmi.ru; Tel.: +7-499-135-03-41

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Abstract: The contamination of marine and freshwater ecosystems with the items from thermoplastics, including polystyrene (PS), necessitates the search for efficient microbial degraders of these polymers. In the present study, the composition of prokaryotes in biofilms formed on PS samples incubated in seawater and the industrial water of a petrochemical plant were investigated. Using a high-throughput sequencing of the V3–V4 region of the 16S rRNA gene, the predominance of *Alphaproteobacteria* (*Blastomonas*), *Bacteroidetes* (*Chryseolinea*), and *Gammaproteobacteria* (*Arenimonas* and *Pseudomonas*) in the biofilms on PS samples exposed to industrial water was revealed. *Alphaproteobacteria* (*Erythrobacter*) predominated on seawater-incubated PS samples. The local degradation of the PS samples was confirmed by scanning microscopy. The PS-colonizing microbial communities in industrial water differed significantly from the PS communities in seawater. Both communities have a high potential ability to carry out the carbohydrates and amino acids metabolism, but the potential for xenobiotic degradation, including styrene degradation, was relatively higher in the biofilms in industrial water. Bacteria of the genera *Erythrobacter*, *Maribacter*, and *Mycobacterium* were potential styrene-degraders in seawater, and *Pseudomonas* and *Arenimonas* in industrial water. Our results suggest that marine and industrial waters contain microbial populations potentially capable of degrading PS, and these populations may be used for the isolation of efficient PS degraders.

Keywords: polystyrene; microbial community; high-throughput sequencing; the 16S rRNA gene; biodegradation; the *sty*-operon

1. Introduction

Polystyrene (PS) is a high-molecular-mass synthetic glassy polymer with a linear structure, which is produced industrially by styrene radical polymerization. PS is a physiologically safe and cheap thermoplastic, which is widely used for the production of disposable tableware, medical equipment, packaging, in the construction industry, etc. Out of 61.8 Mt of plastics produced in Europe in 2018, PS production accounted for ~18 Mt [1]. The large scale of PS production and its resistance to biodegradation results in the contamination of freshwater and marine ecosystems with this plastic

[2–5]. In 2015, the global plastic pollution of the oceans was estimated to weigh between 93,000 and 236,000 metric tons [3]. These results are larger than the global estimates by Eriksen et al. [4] but account for ~1% of the plastic input to the ocean, which weighed 4.8–12.7 million metric tons in 2010 alone, according to Jambeck et al. [5]. A potential environmental risk of contamination of marine and freshwater ecosystems with plastics has been reported [6].

Recently, plastics as habitats for biofilm-forming microorganisms have been a focus of attention [7–9]. Plastics arriving into aquatic ecosystems are colonized by microbial biofilms, containing bacteria, algae, fungi, diatoms, and protozoa, which are embedded in the extracellular polymer matrix [10,11]. Biofilms enhance microbial activity; they protect microorganisms from UV-radiation, high salinity, heavy metals, and antibiotics [10–12]. The composition of biofilms formed on polymer substrates incubated in the North Sea [8], in the Pacific and Atlantic oceans [7], in the Baltic Sea [13–15], in the Mediterranean Sea [16], and in urban freshwater ecosystems [17] has been investigated. Specific microbial communities different from those of the ambient ocean water and from assemblages on natural seston were shown to form on plastic. These communities may be involved in plastic decomposition [14,16,18]. Since the available literature indicates the differences between microbial communities from marine and freshwater habitats, screening for potential plastic degraders specific to these habitats is required for their further biotechnological application.

PS is less susceptible to biodegradation [19,20] than the polymers with hydrolyzed functional groups in the main chain, such as polyethylene terephthalate [21]. Its degradation under natural conditions and the observed decrease in its molecular mass are caused by both biotic and abiotic factors [22]. The carbonyl groups (=C=O) resulting from UV irradiation or oxidation are considered more vulnerable to microbial attacks. The purified hydroquinone peroxidase (EC 1.11.1.7) from a lignin-degrading bacterium, *Azotobacter beijerinckii* HM121, decomposed PS in a two-phase system consisting of dichloromethane and water [23]. The application of whole cells, rather than purified enzymes, is, however, economically preferable. There are only a few reports on PS degradation by *Actinomycetes*, *Arthrobacter*, *E. coli*, *Micrococcus*, *Pseudomonas*, and *Rhodococcus ruber* strains [24–26].

PS degradation involves the colonization of its surface by a microbial community and the subsequent action of the presently unidentified exoenzymes, which degrade the polymer to oligomers and even to monomers [25,27]. Microbial communities or mixed cultures were shown to carry out a more efficient PS degradation than pure microbial cultures [28,29]. A promising PS-degrading strain, *Exiguobacterium* sp. YT2, was isolated from the guts of *Tenebrio molitor* L. larvae, which can feed on foamed PS [30].

A wide range of phylogenetically diverse aerobic bacteria can degrade styrene, the PS monomer [26]. Under oxic conditions, styrene is usually metabolized via side chain oxidation (the "upper pathway") [31,32]. The key enzymes of this styrene-specific pathway are styrene monooxygenase (SMO; 1.14.14.11), which oxidizes styrene to styrene oxide, as well as styrene oxide isomerase (SOI, 5.3.99.7), which transforms it into phenylacetaldehyde. Phenylacetaldehyde dehydrogenase (PAD, 1.2.1.39) converts phenylacetaldehyde to phenylacetic acid, the major intermediate metabolite. Phenylacetic acid is then incorporated into other bacterial metabolic processes via a number of pathways. The functional genes encoding the enzymes of this pathway are organized in the *sty*-operon, in which *styA*/*styB* encodes the two-component styrene monooxygenase, *styC* encodes styrene oxide isomerase, and *styD* encodes phenylacetaldehyde dehydrogenase.

A less common pathway of aerobic styrene degradation begins with oxidation of the aromatic nucleus by styrene 2,3-dioxygenase (1.14.12.-) to styrene *cis*-glycol. This pathway is not specific to styrene and may be used for oxidation of other aromatic compounds, such as ethylbenzene, toluene, and phenol [33]. Anaerobic styrene degradation by microbial associations and members of the genus *Enterobacter* was also shown. However, the pathways of such degradation and the genetic aspects and enzyme systems involved are still insufficiently studied [34].

The goal of the present work was to investigate the phylogenetic diversity of prokaryotes in the biofilms formed on PS surfaces in aquatic media (industrial water and seawater). The putative bacterial community functional profiles were predicted using 16S rRNA amplicon data. In addition, we determined the potential capacity of detected bacteria for styrene degradation using in silico

search for genetic determinants of styrene degradation in the genomes of phylogenetically related bacteria.

2. Materials and Methods

2.1. Incubation of PS Samples

The preparation of PS samples for incubation in aquatic media was carried out using the methods described previously [35]. PS samples (100 × 10 × 2 mm) were immersed into aquatic media near cities in different regions of Russia: Gelendzhik (near Cape Tonkiy, the Black Sea) and Ufa. The samples G10, G40, and G60 (the Marine group) were incubated in August-September 2018 in Black Sea salt water at 1-m depth for 10, 40, and 60 days, respectively. The average, maximal, and minimal temperatures of seawater in the area of Cape Tonkiy were 26.8, 26.0, and 27.8°C, respectively, in August and 25.1, 22.8, and 26.7 °C, respectively, in September 2018. The samples U10, U20, U40, and U60 (the Industrial group) were incubated for 10, 20, 40, and 60 days, respectively, at 14 to 27 °C in the block with recycling industrial water (the Ufaorgsintez petrochemical plant, Ufa). When microbial numbers in recycling water exceeded 10^5 cells/mL, 10% alkaline solution of sodium hypochlorite, an antiseptic widely used for water disinfection, was added to the block [36]. The samples U20, U40, and U60 were subjected to such treatment simultaneously (after 17 days of incubation). Then, the sample U20 was recovered, while the samples U40 and U60 were incubated further, to a total incubation time of 40 and 60 days, respectively.

2.2. Analytical Methods

The chemical composition of the waters was determined with a Biotronic ion chromatograph [37]. Alkalinity, total hardness, and total Fe^{2+}/Fe^{3+} were determined with an Aquamerck alkalinity analytic kit, an Aquamerck Total Hardness kit, and an Iron Test kit (Merck, Darmstadt, Germany), respectively. The values of pH were determined using an Anion ionometer (Novosibirsk, Russia) with relevant electrodes. The determination of the chemical oxygen demand index was performed according to GOST 31859-2012 (Russian State Standard), following the standard procedures by the Russian Federation Gosstandart. The measurements were performed in three replicates.

2.3. Microbial Community Analysis

DNA Extraction: Microbial biomass was washed off the surface of PS specimens with ethanol (1:1, vol/vol) and filtered through membrane filters with 0.22-μm pores (Millipore, Temecula, CA, USA). The filters were then treated with a solution containing 0.15 M NaCl and 0.1 M Na_2EDTA (pH 8.0), and used to isolate DNA. DNA was extracted using the Pure Link Microbiome DNA Purification KIT (Thermo Fisher Scientific, Bartlesville, OK, USA) according to the manufacturer's recommendations.

High-Throughput Sequencing of 16S rRNA Gene Fragments and Their Analysis: DNA isolated from the biofilms grown on PS specimens was used to amplify the V3–V4 hypervariable region of the 16S rRNA gene with the universal Pro341F–Pro805R primer system described by Takahashi et al. [38]. The 16S rRNA gene fragments were amplified using 5× Taq Red buffer and HS Taq polymerase (Evrogen, Moscow, Russia). The reaction mixture contained 5 μL of each primer (6 μM concentration), 5 μL DNA solution, and 15 μL PCR mix (1 U polymerase, 0.2 mM of each dNTP, and 2.5 mM Mg^{2+}). The DNA was amplified using the iCycler thermocycler model from Bio-Rad (Hercules, CA, USA). The PCR reaction conditions for DNA amplification were as recommended by Takahashi et al. [38]: initial denaturation at 98 °C for 2 min, followed by 35 cycles of annealing beginning at 65 °C and ending at 55 °C for 15 s, and elongation at 68 °C for 30 s. The annealing temperature was lowered 1 °C for every cycle until reaching 55 °C, and then used for the remaining cycles. Fragments of the 16S rRNA gene were amplified on the template of DNA isolated from each specimen in three replicates, which were subsequently combined and purified by electrophoresis in a 2% agarose gel using a Cleanup Standard gel extraction kit (Evrogen, Russia). High-throughput

sequencing was performed using the MiSeq system (Illumina, San Diego, CA, USA) with a MiSeq Reagent Kit v3 (600 cycles) (Illumina), as recommended by the manufacturer.

The reads were further processed according to the following workflow, implementing suitable scripts from USEARCH v.10 [39]. Pair-end reads were demultiplexed (-fastx_demux), merged (-fastq_mergepairs), trimmed in order to remove the primer sequences (-fastx_truncate), and then quality-filtered (-fastq_filter). UNOISE3 [40] was used to generate zero radius operational taxonomic units (OTUs). Zero radius OTU is a term specific to analysis with UNOISE, referring to operational taxonomic units which were generated by an error correction algorithm as opposed to a sequence similarity clustering algorithm [41]. Raw merged read pairs were mapped back to OTUs using the -otutab command. Zero radius OTUs were submitted for taxonomic analysis in the SILVA database (SINA, https://www.arb-silva.de/aligner/, April 2020, version 1.2.11 [42], SILVA reference database release 138) using default settings. The OTUs that could not be assigned to the *Bacteria* or *Archaea* were discarded prior to the analyses. These OTUs were excluded manually based on the SILVA classification. The alpha-diversity and beta-diversity of the bacterial communities were estimated by -alpha_div and -beta_div workflows. Rarefaction curves were obtained using the -alpha_div_rare command. The beta-diversity of taxon abundance tables was visualized through the non-metric multidimensional scaling (NMDS) of Bray-Curtis distances using the "metaMDS" function in the R package Vegan version 2.5–6 (https://cran.ism.ac.jp/web/packages/vegan/vegan.pdf, 1 September 2019) [43]. Permutational ANOVA (PERMANOVA), using the PRIMER 7 PERMANOVA+ software [44], was applied to test for differences in microbial community composition (Bray-Curtis distances) between the studied locations, using a one-factor design with the site (Marine or Industrial group) as fixed factor and the incubation times as replicates. Venn diagrams were constructed using the online tool InteractiVenn [45].

The iVikodak software package [46] was applied to predict the functional characteristics of bacterial communities, using a phylogenetic analysis of the 16S amplicon data, generated from the studied samples. The list of genera for all 16S rRNA gene libraries based on the OTUs table (% of total reads for each library) was provided as input in iVikodak. The Global Mapper module was used for inferring functional profiles, and Local Mapper was used for predicting individual pathway enzyme profiles using the KEGG pathway database. Pathway exclusion cut-off (PEC) was set at 80 to strengthen the confidence of the data. Relevant heatmaps based on the results of the metabolic inference on genus level were constructed using the ClustVis [47] online resource (http://biit.cs.ut.ee/clustvis/, April 2020). The search for the genes of styrene degradation was carried out using the KEGG (https://www.genome.jp/kegg/pathway.html, release 94.0, 1 April 2020), PATRIC (https://www.patricbrc.org, version 3.6.3, September–December 2019), and NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi, BLAST+ 2.10.0, 16 December 2019) online databases and services. The gene cluster of *sty*-operon in genomes was compared using the Gene Graphics tool [48].

2.4. Scanning Electron Microscopy (SEM)

The biofilms on the surface of PS samples were fixed, dehydrated, and coated with gold as described previously [49]. Bacterial biofilms and the surface of PS specimens were investigated using a VEGA 3 XMU scanning microscope (TESCAN, Brno, Czech Republic) and the ADVANCED AZTEC Energy system of energy dispersion microanalysis (including Inca Energy 350) based on an X-MAX 50 STANDARD EDS detector (Oxford Instruments Nano-Analysis, Oxford, UK) at an acceleration voltage of 2 kV.

2.5. Nucleotide Sequence Accession Numbers

The obtained libraries of 16S rRNA gene fragments from bacterial biofilms of PS specimens were deposited into the NCBI SRA database with the accession number SRR11344280–SRR11344287.

3. Results

3.1. Physicochemical Characteristics of Aqueous Environments

The total salinity and hardness of the recirculated water of the petrochemical plant were lower than in the seawater (Table 1). Temperature variations at the incubation sites in the marine basin were caused by diurnal fluctuations and were within the range of the temperatures for industrial recycling water. The chemical oxygen demand (COD) of industrial water decreased in the course of incubation from 103 to 72 mg O_2/dm^3, which indicated a gradual removal of harmful contaminants from the recycling water during this period. The seawater and industrial water samples were alkaline (pH 7.8–8.5).

Table 1. Chemical composition of seawater and industrial water used for incubation of polystyrene (PS) samples.

Habitat, Specimen	Total Salinity, mg/L	pH	Water Temperature, °C	Total Hardness, mg-eq/L	Total Iron, mg/L	Alkalinity, mg-eq/kg	COD [1], mg O_2/dm^3
Industrial Water							
U10	1098	8.5 ± 0.1	24.0–27.0	13.8	0.45	6.4	101
U20	1100	8.5 ± 0.1	21.5–25.5	12.6	0.37	6.5	103
U40	918	8.4 ± 0.1	19.0–23.0	9.8	0.21	4.3	72
U60	676	8.1 ± 0.1	14.0–18.0	6.7	0.61	2.4	90
Black Sea [2], G10, G40, G60	18,600	7.8 ± 0.1	26.8–25.1	66.9	Nd [3]	Nd	Nd

[1] COD, chemical oxygen demand. [2] All data on the seawater, except water temperature, were obtained at the beginning of the incubation period. [3] Nd, no data.

3.2. Analysis of Bacterial Diversity in Biofouling Colonizing PS Specimens in Aqueous Environments

High-throughput sequencing of the V3–V4 region of the 16S rRNA gene resulted in a total of 248,776 16S rRNA gene fragments (reads) for the libraries from seven samples of microbial biofilms growing on PS. For the molecular studies, we used only one PS specimen at each time of sampling. Thus, combining all samples across time within a site, we carried out a comparison between the two sites. The sequences were grouped into 1372 operational taxonomic units (OTUs). The numbers of detected OTUs, alpha diversity, and rarefaction curves data obtained after amplicon sequencing are presented in Appendix A, Figure A1. The rarefaction curves were saturated after 5–10 thousand reads, indicating that sufficient coverage was obtained in this study. Diversity indices for the libraries from both incubation sites are shown in Table 2.

Table 2. Diversity indices in the 16S rRNA gene libraries of bacterial communities on the surface of PS specimens exposed to water environments.

Parameter	Libraries of 16S rRNA Gene Fragments						
	Industrial				Marine		
	U10	U20	U40	U60	G10	G40	G60
Duration of exposure, days	10	20	40	60	10	40	60
Number of sequences (reads)	29,466	56,405	37,552	29,546	27,243	24,261	44,303
Number of OTUs	474	703	525	483	558	443	569
Shannon–Weaver diversity index (*H*)	3.5	5.7	4.6	4.1	5.2	4.3	5.2
Inverse Simpson diversity index (1/*S*)	8.1	138.9	32.6	17.1	68.5	14.0	54.6
Evenness (*H*/*H*max)	0.56	0.87	0.72	0.67	0.82	0.71	0.81
Berger–Parker dominance index (*D*)	0.32	0.04	0.10	0.19	0.06	0.21	0.09

For the libraries of the samples incubated in the Black Sea water (the Marine group) and in the industrial water (the Industrial group), the Shannon–Weaver indices were similar. Biofilms of the Industrial group had the lowest values of diversity indices and the highest value of the dominance index in sample U10. The highest diversity indices were found in sample U20, which was incubated

for 20 days. The Shannon–Weaver and Simpson indices from the samples collected after antimicrobial chemical treatment decreased till the end of incubation. The comparison of the OTUs in the libraries using Venn diagrams gives a dynamic visual representation of changes in microbial diversity on PS in industrial water and seawater (Appendix A, Figure A2a,b). The analysis of beta-diversity using Bray–Curtis dissimilarity and multivariate analysis (PERMANOVA) revealed significant differences in the bacterial community structure of Marine and Industrial groups (Figure 1, PERMANOVA: 999 permutations, pseudo F = 3.62, *p*-value = 0.04).

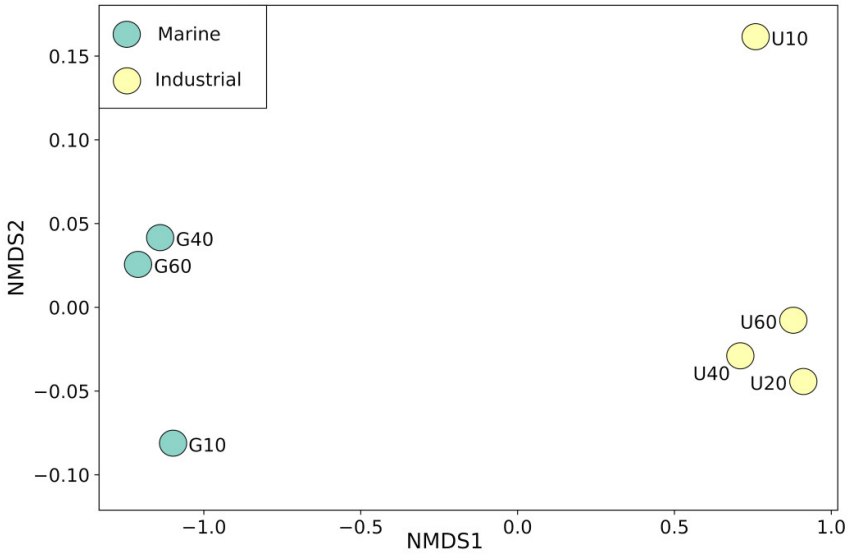

Figure 1. Non-metric multidimensional scaling (NMDS) plot based on the Bray–Curtis similarity coefficients of marine and industrial bacterial communities. Points closer to one another in ordination space are more similar than those apart.

3.3. Prokaryotes Phylogenetic Diversity in Biofilms Formed on PS Samples

As a result of the high-throughput sequencing of the 16S rRNA genes, only members of the *Bacteria* domain were found in the studied biofilms, while *Archaea* were not detected. At the phylum/class level, the communities were dominated by members of *Alphaproteobacteria* (14.4–63.6% of the total number of sequences in the library), *Gammaproteobacteria* (8.5–70.5%), *Bacteroidota* (2.5–20.9%), *Actinobacteriota* (2.4–6.3%), *Planctomycetota* (0.3–11.6%), and *Verrucomicrobiota* (0.4–7.2%) (Figure 2; Appendix A, Table A1).

The taxonomic composition of the 50 most relative abundant genera varied both from group to group and between the libraries of each group (Figure 3; Appendix A, Table A1). In the U10, community bacteria of the genus *Blastomonas* (*Alphaproteobacteria*) (40.7%) predominated. In the course of longer incubation (samples U20–U60), the community composition changed in favor of members from the genera *Chryseolinea* (*Bacteroidetes*), *Arenimonas*, and *Pseudomonas* (*Gammaproteobacteria*). In the Marine group communities, during the incubation, the proportion of the genus *Erythrobacter* (*Alphaproteobacteria*) increased and that of the genera *Pelagicoccus* (*Verrucomicrobiota*), *Pseudohongiella* (*Gammaproteobacteria*), and *Planctomicrobium* (*Planctomycetota*) decreased.

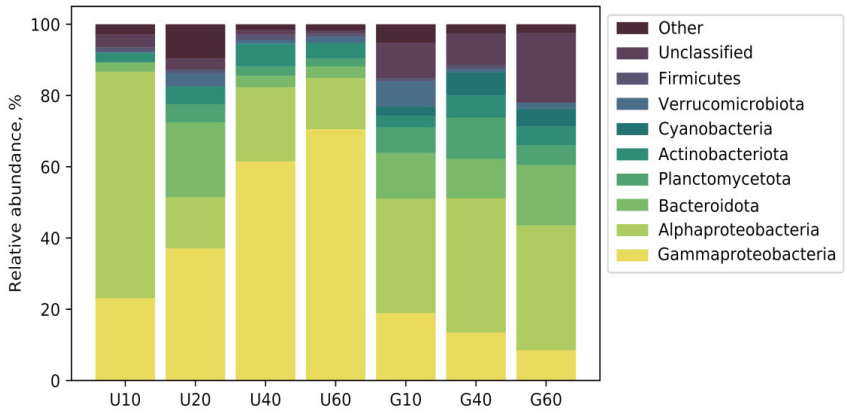

Figure 2. Relative proportion of bacterial 16S rRNA gene sequences from the PS samples presented at the phylum/class level. The taxa constituting at least >1% in each library are listed.

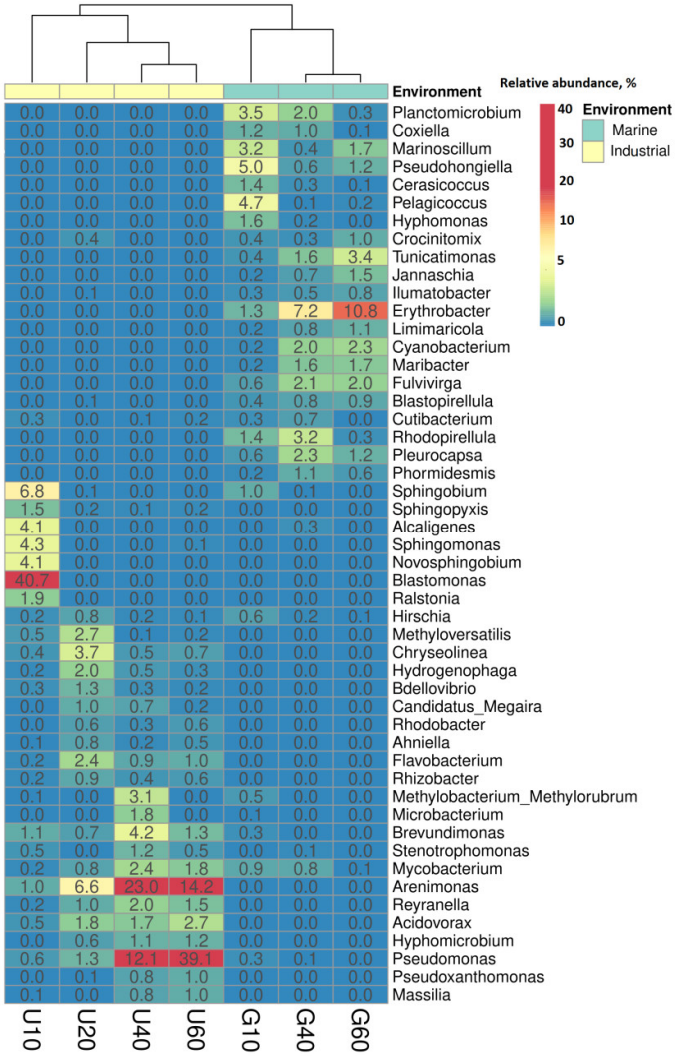

Figure 3. Heatmap of the distribution of 50 most relative abundant genera in the libraries of the 16S rRNA gene sequences from PS specimens. The representation of the genus was calculated by dividing the sequence proportions by the total sequence count in each library. Columns are clustered using correlation distance and average linkage.

3.4. Potential Ability of the Studied Bacterial Communities to Degrade Styrene

Our results on bacterial phylogenetic diversity in the libraries were analyzed using the Global Mapper module of the iVikodak software in order to assess the potential contribution of the studied communities to the metabolic pathways.

Function abundance profiles for the studied communities are shown in Appendix A, Figure A3 and Figure 4. Carbohydrate metabolism, followed by amino acid metabolism, replication and repair, energy metabolism, and translation were revealed in all samples; however, xenobiotics biodegradation and metabolism were relatively pronounced in samples U10 and U60 (Appendix A, Figure A3). We found that the communities of the Industrial group (U10–U60) exhibited the highest potential functional diversity and ability to carry out most pathways of xenobiotic degradation, including the degradation of styrene, the PS monomer ("Styrene degradation"), and the incorporation of phenylacetic acid, the major metabolite of styrene degradation ("Phenylalanine metabolism") (Figure 4).

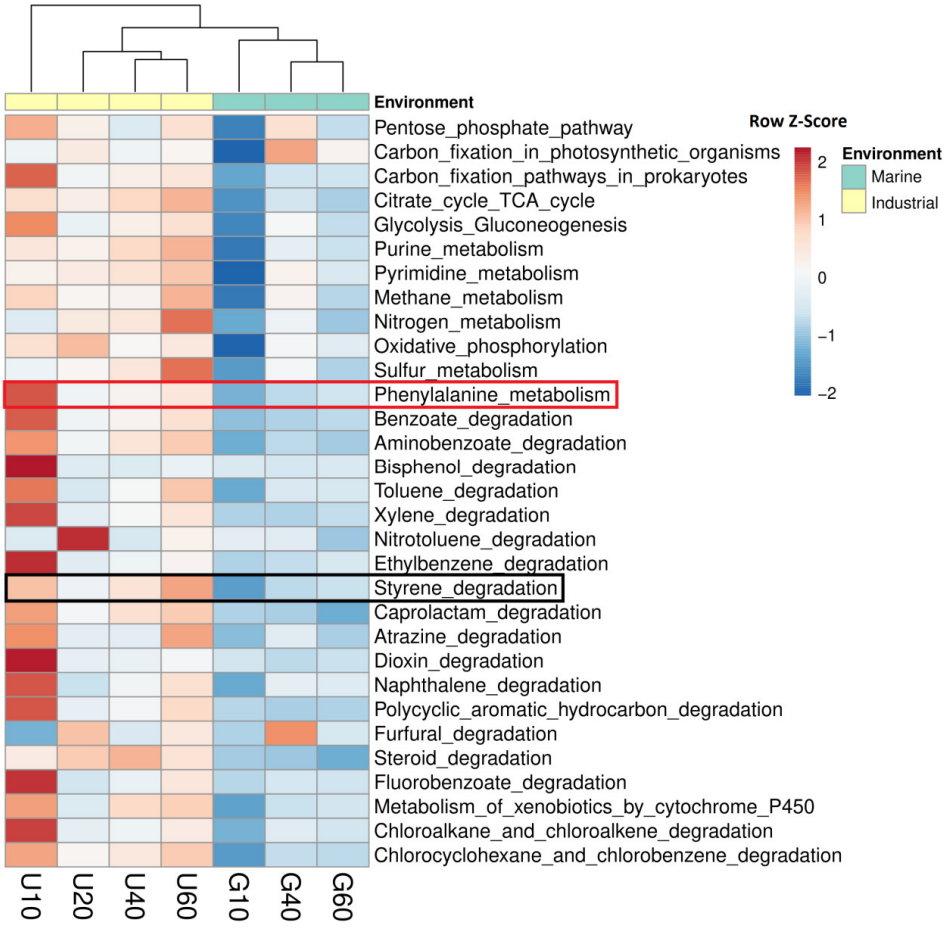

Figure 4. The heatmap showing the clustering of PS samples based on the relative percentage of iVikodak-derived bacterial community functional profiles. The later analyzed styrene and phenylalanine metabolic pathways are marked.

The Local Mapper module of the iVikodak package was used to analyze the representation of the enzymes of the "Styrene degradation" pathway in members of the bacterial communities (Appendix A, Figure A4). This analysis predicted two key enzymes for aerobic styrene degradation in bacterial communities of the Industrial group, which were responsible for styrene catabolism to phenylacetaldehyde, as well as for phenylacetaldehyde dehydrogenase, which converts phenylacetaldehyde to phenylacetic acid, the key intermediate ("upper pathway") (Appendix A,

Figure A5a). Phenylacetaldehyde dehydrogenase was better represented, since it is not specific to styrene degradation and is also involved in phenylalanine metabolism ("Phenylalanine metabolism") (Appendix A, Figure A5b). The program did not reveal the key enzyme of another pathway for styrene degradation, styrene 2,3-dioxygenase, catabolizing styrene conversion to styrene cis-2,3-dihydrodiol. The same iVikodak module may be used to assess the contribution of various bacterial taxa to the analyzed metabolic pathways. Members of the genera *Pseudomonas*, *Arenimonas*, and *Acidovorax* had the greatest functional potential for styrene degradation in the industrial water (Appendix A, Figure A6a), and those of *Erythrobacter*, *Maribacter*, and *Mycobacterium* in seawater (Appendix A, Figure A6b). The assignment of the genera to *Betaproteobacteria*, as shown in Appendix A, Figure A6 (iVikodak software), does not coincide with their affiliation to high taxon presented in Appendix A, Table A1, which was constructed using the SILVA database. This is the result of the application of the RDP classifier in iVikodak. The problems of differentiation among the higher taxa are, however, beyond the scope of the present work.

3.5. Identification of Bacteria Possessing sty-Operon Genes

In the "KEGG organisms in the NCBI taxonomy" database only a single styrene-degrading strain, *Pseudomonas* sp. VLB120 [50,51], was associated with SMO and SOI enzymes. Therefore, the genes of *sty*-operon of this strain were used for searching other bacterial genomes possessing the genes of *sty*-operon in the PATRIC genome database. The PATRIC protein families contain similar proteins believed to perform the same function. They come in two flavors—global families that spread across all genomes and local families restricted to a single genus. As a result of the comparison of global families for each gene of *sty*-operon, strains for which the presence of complete *sty*-operon was most likely were selected. Most of the selected strains belonged to the genus *Pseudomonas*. However, the *styA* gene as well as homologs of the *styB* and *styC* genes (although not always annotated) were also found in members of the genera *Altererythrobacter*, *Amycolatopsis*, *Bradyrhizobium*, *Burkholderia*, *Glaciecola*, *Marinobacter*, *Marinobacterium*, *Mycobacterium*, *Nocardia*, *Panacagrimonas*, *Pseudorhodoplanes*, *Rhodococcus*, *Rhodopseudomonas*, *Sorangium*, *Sphingobium*, *Sphingopyxis*, *Tropicibacter*, and *Zavarzinia*. Some reference strains belonging to these genera were chosen for further analysis of *sty*-operon (Appendix A, Figure A7). The 16S rRNA genes of these bacteria were used for detection of related OTUs with at least 99% homology in the libraries obtained. In addition, the identification of these OTUs was further verified using the BLAST module of the Genbank online platform. A total of 14 OTUs were revealed (Appendix A, Table A2). The taxonomic position and proportion of the OTUs potentially representing styrene-degrading bacteria varied for PS samples incubated in different aquatic environments. In the Industrial group, most OTUs belonged to the genera *Pseudomonas* (related to *P. putida*, *P. aeruginosa*, and *P. stutzeri*), *Rhodococcus*, *Sphingopyxis*, and *Nocardia*. In the Marine group, potential styrene degraders belonged to the genera *Altererythrobacter*, *Mycolicibacterium*, *Pseudomonas*, and *Tropicibacter*.

3.6. Microscopy of Bacterial Biofilms on PS

Microbial biofilms formed on PS samples incubated in marine and industrial environments and the samples with washed-off biofilms were studied by scanning electron microscopy. Microbial growth on the sample U10 (the Industrial group), which was incubated for the shortest time and was not subject to antimicrobial treatment, took the form of loose conglomerates of bacterial colonies with few structured microeukaryotes. Neither the biofilm nor the precipitates of calcium salts formed in mineralized water covered the entire surface of the sample, so that areas of clean PS could be seen (Appendix A, Figure 5a). The PS samples U40 and U60 were covered with biofilms due to long-term incubation (Appendix A, Figures 4c and 5b). The local degradation of the PS surface may be seen on the photograph of the U60 sample with washed-off biofilm (Appendix A, Figure 5d).

The microbial biofilms on the Marine group samples were different from those of the Industrial group. The biofilm was more diverse, covering almost all the sample surface even at the early term of incubation (Appendix A, Figure 5e–g). Microeukaryotes morphologically similar to diatoms were

present in biofilms (Appendix A, Figure 5g). The small-scale degradation of the PS surface was visible on the PS sample G60 with the longest time of incubation (Appendix A, Figure 5h).

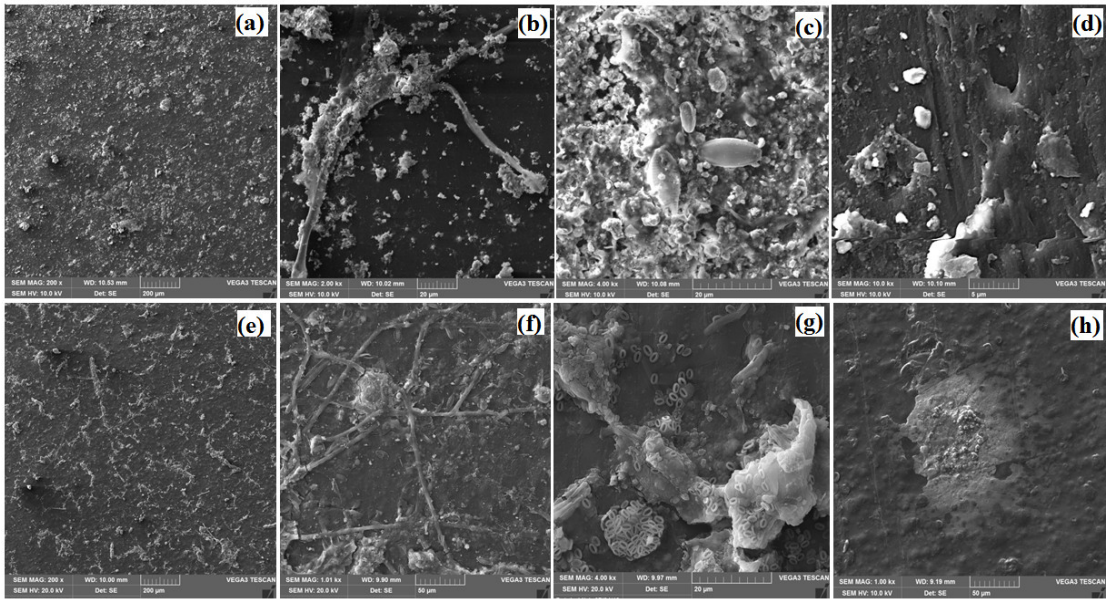

Figure 5. Scanning electron microscope (SEM) images of biofilms on PS specimens after 10 days (U10, **a**), 40 days (U40, **b**), and 60 days (U60, **c**) of incubation in industrial water, and SEM image showing the surface modifications of specimen U60 after removal of the biofilm (**d**). SEM images of biofilms on PS specimens after 10 days (G10, **e**), 40 days (G40, **f**), and 60 days (G60, **g**) of incubation in seawater, and SEM image showing the surface modifications of specimen G60 after removal of the biofilm (**h**).

4. Discussion

Microbial communities involved in the degradation of PS and other polymers are relatively poorly studied [13,23,27,52]. The investigation of microbial communities colonizing polymers in natural environments is a necessary step for further screening of efficient polymer degraders. In this study, we showed that PS samples exposed to seawater of the Black Sea and industrial water of a petrochemical plant were colonized by different bacterial communities with different potential functions. By scanning electron microscopy, biofilms were observed, as well as the local degradation of the PS surface after 60 days of incubation (Figure 5). Bacterial assemblages on PS samples were analyzed using 16S rRNA-gene amplicon sequencing and in silico functional characterization.

The differences revealed between the Marine and Industrial groups' communities formed on PS samples may be caused by both environmental physicochemical parameters (aggressive petrochemical waste in industrial water and high salinity and UV radiation in seawater) and the effect of antimicrobial treatment of industrial water. Marine and industrial communities on PS samples were clustered separately (Figure 1). Members of the classes *Alphaproteobacteria* (*Sphingomonadales*), *Bacteroidetes*, and *Gammaproteobacteria* were the major groups in the studied biofilms on PS immersed in seawater (G10, G40, G60). Our results correlated with the published data. Members of *Gammaproteobacteria*, *Alphaproteobacteria*, and *Bacteroidetes* dominated also in natural marine biofilms and in biofilms on various plastic materials exposed in seawater [12,52–54]. *Alphaproteobacteria* were the key primary surface colonizers in marine environments [54–57]. Within the *Alphaproteobacteria*, different genera of *Rhodobacteraceae* [54] and *Sphingomonadales* (*Altererythrobacter* and *Erythrobacter*) [58] are often found in marine biofilms. These bacteria were also present in biofilms formed on polyethylene terephthalate (PET) samples exposed to the Black Sea water [35] and on PET and PS samples incubated in the Baltic Sea [14]. It was hypothesized in the latter work that *Erythrobacter* catabolizes not the polymer as such, but rather the associated polycyclic aromatic hydrocarbons sorbed from the aquatic environment. *Bacteroidetes* (mainly the *Flavobacteria*) were recognized as an

important group of secondary surface colonizers in marine environments [59]. *Planctomycetes* (*Rhodopirellula*, *Planctomicrobium*) and *Verrucomicrobia* (*Pelagicoccus*) revealed in the studied G10–G60 samples are typical inhabitants of marine ecosystems. These bacteria are known to form associations with multicellular eukaryotes (macroalgae and invertebrates) [60]. It was suggested that bacteria inhabiting plastic surfaces in seawater possess the metabolic potential to degrade plastics and/or plastic-bound organic pollutants [8,16,18]. This conclusion was supported by the KEGG analysis of metagenomics data, which revealed a high xenobiotic degradation potential of plastic-associated communities in the North Pacific Gyre [61]. In seawater-exposed samples, physicochemical PS degradation due to UV radiation probably occurred as well. Polymer decomposition caused by physicochemical factors and the products of microbial metabolism at the first stages of incubation could promote the activity of bacterial strains able to use styrene in their metabolism.

Taxonomic analyses confirmed the specificity of the community structures formed on the PS samples incubated in industrial water compared to seawater. *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, and *Bacteroidetes* predominated in Industrial PS samples (Figure 2). PS samples were first colonized by *Alphaproteobacteria* of the family *Sphingomonadaceae*, including members of the genera *Blastomonas* (40.7%), *Sphingobium* (6.8%), *Sphingomonas* (4.3%), and *Novosphingobium* (4.1%); their share decreased greatly with time (lower than 1%) (Figure 3; Appendix A, Table A1). Bacteria of the genus *Blastomonas* are known to produce adhesins in freshwater environments, forming biofilms together with other bacteria [62]. Although adhesion intensifies in the presence of bacteria producing adhesins and exopolysaccharides [63], this process is known to be weak and reversible [24] and results in a shift in the composition of biofilm microbial communities.

Hypochlorite treatment of industrial water, which was a part of the technological process, affected the community composition, as was evidenced by the community of the U10 PS sample (untreated with hypochlorite) occupying a separate position relative to the U20–U60 samples (Figure 1). However, the present work focused on the detection of potential styrene degraders in seawater and industrial water, rather than on the determination of hypochlorite's effect on the community composition. Antimicrobial treatment of industrial water with sodium hypochlorite resulted in a shift in the composition of dominant bacteria to *Arenimonas* and *Pseudomonas* (*Gammaproteobacteria*), which were probably more resistant to the reagent. The cyclic formation and removal of biofilms resulted in a more intense physicochemical degradation of the polymer, which contributed to its biodegradation by microorganisms [36]. Moreover, the presence of other aggressive reagents in industrial water, which affected the polymer structure, probably also contributed to PS chemical degradation. At later stages of incubation of PS samples in industrial water, the community composition continued to change, which probably correlated with the enhanced biodegradation and accumulation of degrader strains in the biofilm.

Based on the 16S rRNA gene data, we predicted the functional profiles of the studied communities. Community composition differed between the industrial and marine sites, and there were concomitant variations in the functional potential. Although carbohydrate and amino acid metabolism was potentially high both in industrial and marine communities, the potential for the degradation of xenobiotics, including styrene, was at higher proportions in the industrial group (Figure 4; Appendix A, Figure A3–5). Our analysis of the genomic database indicates the presence of the *sty*-operon genes in the genomes of bacteria of the genera *Pseudomonas*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, *Marinobacterium*, and *Sphingopyxis*, whose ability to degrade styrene was confirmed [26,53]. For a number of reference strains used in this work to search for similar sequences in our libraries, data are available not only on the ability to metabolize styrene, but also on the activity and structure of the relevant enzymes. This information exists for the strains *Sphingopyxis* sp. Kp5.2 [64], *Rhodococcus opacus* 1CP [65], *P. putida* CA-3 [66], *Pseudomonas* sp. LQ26 [67], *Pseudomonas* sp. VLB120 [50], and *P. putida* S12 [68]. It may be suggested that bacteria represented in our libraries by the OTUs with the highest similarity of their 16S rRNA gene sequences (>99–100%) to those of styrene-degrading reference bacteria (Appendix A, Table A2, Figure A7) are the most probable potential styrene degraders. In the industrial water, potential styrene degraders belonged to the genera

Pseudomonas, *Arenimonas*, *Acidovorax*, and *Mycobacterium*, and in the seawater to the genera *Erythrobacter*, *Maribacter*, and *Mycobacterium* (Appendix A, Figure A6).

It is known that in several *Pseudomonas* strains the *sty*-operons are located on the chromosome and on a plasmid, and that in *Sphingopyxis fribergensis* Kp5.2 it is located only on the chromosome [26,64]. In *Pseudomonas* sp. VLB120, the chromosomal *sty*-operon is flanked by a mobile element and is probably involved in lateral transfer of the *styABC* genes (Appendix A, Figure A7). The lateral transfer of the genes of the *sty*-operon may promote the propagation of the styrene-degrading capacity in aquatic environments. The results indicate that the biofilms formed on PS and other plastics in seawater or in petrochemical-contaminated industrial water may be useful microbial resources for the isolation of efficient plastic degraders.

5. Conclusions

Plastic contamination is a worldwide problem and needs an effective solution. The biofilms formed on PS samples in marine and industrial aqueous environments contained different microbial communities. Members of *Alphaproteobacteria*, *Gammaproteobacteria*, and *Bacteroidetes* predominated in biofilms on PS in both aquatic habitats, while *Planctomycetes* were better represented in seawater. The studied marine and industrial communities on PS were particularly enriched in carbohydrate and amino acid metabolism pathways genes, while the genes of xenobiotic degradation and styrene degradation pathways were relatively enriched in bacterial assemblages in industrial water. Putative styrene-degrading bacteria belonging to the genera *Pseudomonas*, *Arenimonas*, *Acidovorax*, and *Mycobacterium* were detected in the industrial water PS biofilms, and bacteria of the genera *Erythrobacter*, *Maribacter*, and *Mycobacterium* in seawater PS biofilms. Future work should screen the capacity of these taxa for styrene degradation. These results suggest that marine and industrial waters contain microbial populations potentially capable of degrading PS specimens. The biofilms formed on PS samples incubated in seawater and industrial water may be used for the isolation of biotechnologically valuable PS-degrading bacterial strains.

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Appendix A

Table A1. Relative proportion of the phyla and genera of the *Bacteria* domain in the libraries [1] of the 16S rRNA gene sequences from the biofilms on PS samples incubated in seawater and industrial water.

Taxonomic Group, Genus	Industrial				Marine		
	U10	U20	U40	U60	G10	G40	G60
Actinobacteriota	2.45	4.88	6.26	4.33	3.25	6.27	5.23
Rhodococcus	0.01	0.01	1.19	0.04	0.04	0.00	0.01
Microbacterium	0.05	0.00	1.82	0.03	0.11	0.01	0.00
Mycobacterium	0.15	0.83	2.37	1.78	0.87	0.85	0.09
Alphaproteobacteria	63.57	14.41	20.76	14.41	32.07	37.65	35.04
Jannaschia	0.00	0.00	0.00	0.00	0.18	0.65	1.53
Hyphomonas	0.01	0.03	0.04	0.02	1.56	0.19	0.03
Limimaricola	0.00	0.00	0.00	0.00	0.24	0.81	1.15
Ca. Megaira	0.03	1.02	0.74	0.22	0.03	0.00	0.02
Erythrobacter	0.03	0.03	0.03	0.00	1.26	7.20	10.82
Methylobacterium	0.08	0.00	3.09	0.00	0.52	0.01	0.01
Reyranella	0.17	0.97	1.96	1.47	0.00	0.00	0.00
Brevundimonas	1.06	0.67	4.21	1.35	0.35	0.00	0.00
Sphingopyxis	1.51	0.17	0.13	0.17	0.01	0.00	0.01
Novosphingobium	4.12	0.02	0.00	0.01	0.02	0.00	0.00
Sphingomonas	4.29	0.00	0.00	0.12	0.00	0.00	0.01
Sphingobium	6.81	0.06	0.04	0.02	1.05	0.08	0.00
Blastomonas	40.74	0.04	0.00	0.01	0.01	0.04	0.03
Hyphomicrobium	0.02	0.61	1.09	1.20	0.01	0.00	0.00
Gammaproteobacteria	23.09	37.08	61.53	70.54	18.95	13.49	8.51
Pseudomonas	0.55	1.31	12.11	39.10	0.33	0.09	0.01
Arenimonas	1.02	6.59	23.04	14.17	0.02	0.00	0.00
Hydrogenophaga	0.19	1.98	0.55	0.30	0.00	0.00	0.03
Stenotrophomonas	0.46	0.01	1.22	0.52	0.04	0.14	0.00
Methyloversatilis	0.48	2.66	0.06	0.19	0.00	0.00	0.00
Acidovorax	0.47	1.82	1.73	2.75	0.00	0.00	0.00
Dechloromonas	1.27	0.10	0.01	0.10	0.00	0.00	0.00
Ralstonia	1.90	0.00	0.00	0.00	0.00	0.00	0.00
Alcaligenes	4.08	0.00	0.00	0.00	0.00	0.27	0.00
Pseudohongiella	0.01	0.00	0.00	0.00	5.02	0.65	1.17
Coxiella	0.00	0.00	0.00	0.00	1.16	0.99	0.08
Planctomycetota	0.31	5.10	2.71	2.26	7.09	11.59	5.54
Rhodopirellula	0.00	0.00	0.00	0.00	1.40	3.17	0.35
Planctomicrobium	0.00	0.04	0.00	0.00	3.54	2.01	0.25
Verrucomicrobiota	0.39	3.82	1.00	1.66	7.18	0.80	1.70
Pelagicoccus	0.01	0.00	0.00	0.00	4.70	0.11	0.16
Cerasicoccus	0.01	0.00	0.00	0.00	1.39	0.31	0.10
Bacteroidota	2.49	20.95	3.26	3.18	12.89	11.06	17.00
Marinoscillum	0.02	0.00	0.00	0.00	3.22	0.37	1.73
Maribacter	0.00	0.00	0.00	0.00	0.22	1.63	1.72
Fulvivirga	0.00	0.00	0.00	0.00	0.61	2.13	1.99
Tunicatimonas	0.01	0.00	0.00	0.00	0.42	1.56	3.38
Taeseokella	0.00	0.00	0.00	0.00	1.10	0.00	0.04
Flavobacterium	0.24	2.36	0.91	1.02	0.00	0.00	0.00
Chryseolinea	0.42	3.69	0.51	0.71	0.00	0.01	0.00

[1] The taxonomic groups responsible for >1% of the total number of bacterial sequences in each library are listed.

Table A2. Proportion of sequences in the operational taxonomic units (OTUs) having >99% similarity to the 16S rRNA genes of the reference strains containing the *sty*-operon genes in their genomes.

OTU	Hit Taxon Name	Hit Strain Name	Accession Number	Similarity, %	% of Total Reads in Each Library						
					Marine			Industrial			
					G10	G40	G60	U10	U20	U40	U60
OTU121	*Pseudomonas aeruginosa*	JCM 5962^T	BAMA01000316	100	0.05	0.01	0	0	0.01	0.03	1.69
OTU23	*P. putida*	NBRC 14164^T	AP013070	99.8	0	0	0	0.05	0	1.38	3.39
OTU4	*P. putida*	NBRC 14164^T	AP013070	100	0	0	0	0.07	0.01	7.99	23.29
OTU46	*P. stutzeri*	ATCC 17588^T	CP002881	100	0.22	0.08	0.01	0.06	0.02	0.04	5.81
OTU131	*Rhodococcus erythropolis*	NBRC 15567^T	BCRM01000055	100	0	0	0	0.004	0.01	0.83	0.02
OTU867	*Sphingopyxis sp.*	113P3	CP009452	100	0	0	0	0	0.05	0.01	0.03
OTU167	*S. italica*	SC13E-S71^T	HE648058	99.7	0.01	0	0.01	0.59	0.01	0	0.004
OTU550	*S. alaskensis*	RB2256^T	CP000356	99.5	0	0	0	0.01	0.05	0.06	0.04
OTU606	*S. fribergensis*	Kp5.2^T	CP009122	100	0	0	0	0	0.04	0.05	0.09
OTU362	*Mycolicibacterium hippocampi*	BFLP-6^T	FN430736	100	0.43	0.49	0.04	0.01	0.004	0	0.004
OTU71	*Nocardia asteroides*	NBRC 15531^T	BAFO01000006	100	0	0	0	0	0.64	0.15	0.11
OTU66	*Altererythrobacter spongiae*	HN-Y73^T	MG437235	99	0.36	0.37	0.62	0	0	0	0
OTU878	*Tropicibacter naphthalenivorans*	CECT 7648^T	CYSE01000022	99	0.12	0.03	0.14	0	0	0	0
OTU1301	*Marinobacter flavimaris*	SW-145^T	NR_025799	99.6	0.018	0	0	0	0	0	0

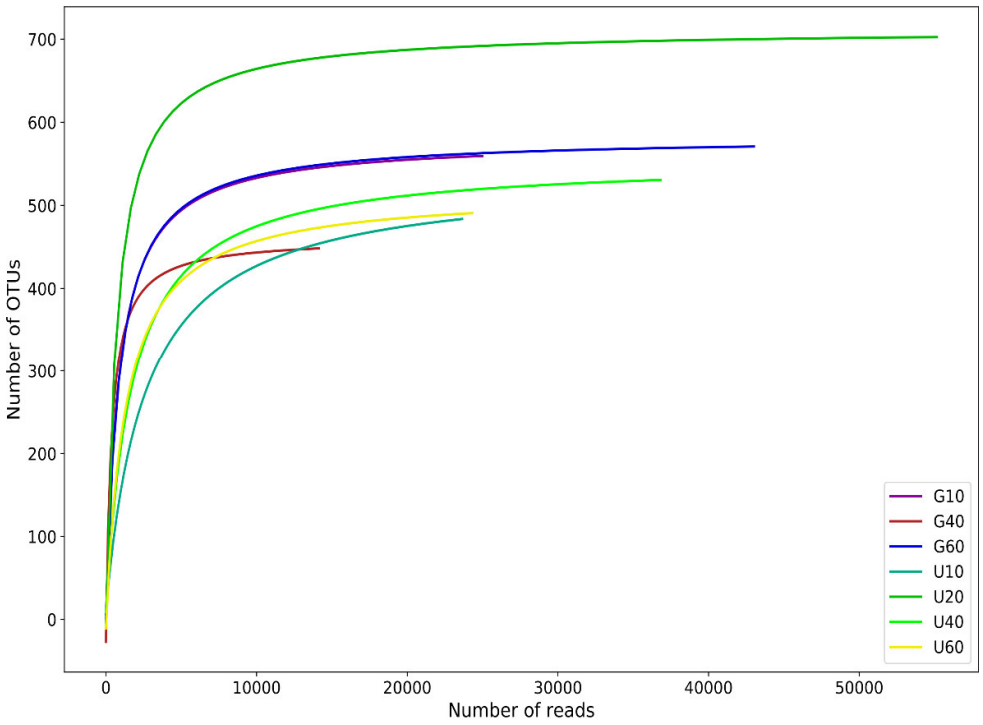

Figure A1. Detected OTUs, alpha diversity, and rarefaction curves data obtained after amplicon sequencing of 16S rRNA genes of bacteria from PS samples incubated in seawater and industrial water.

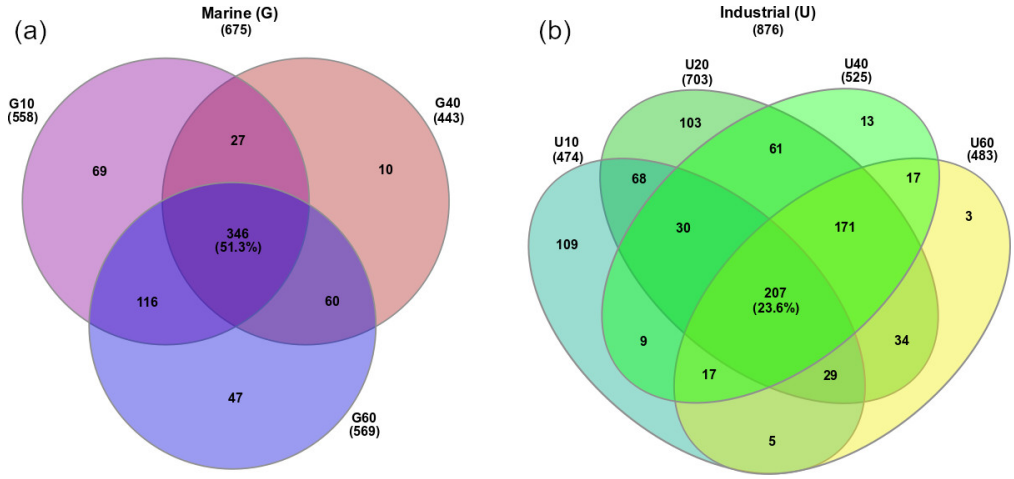

Figure A2. Venn diagrams showing the number and proportion of shared and unique OTUs between the libraries of bacterial 16S rRNA genes on PS samples incubated in seawater (the Marine group, (**a**) and industrial water (the Industrial group, (**b**).

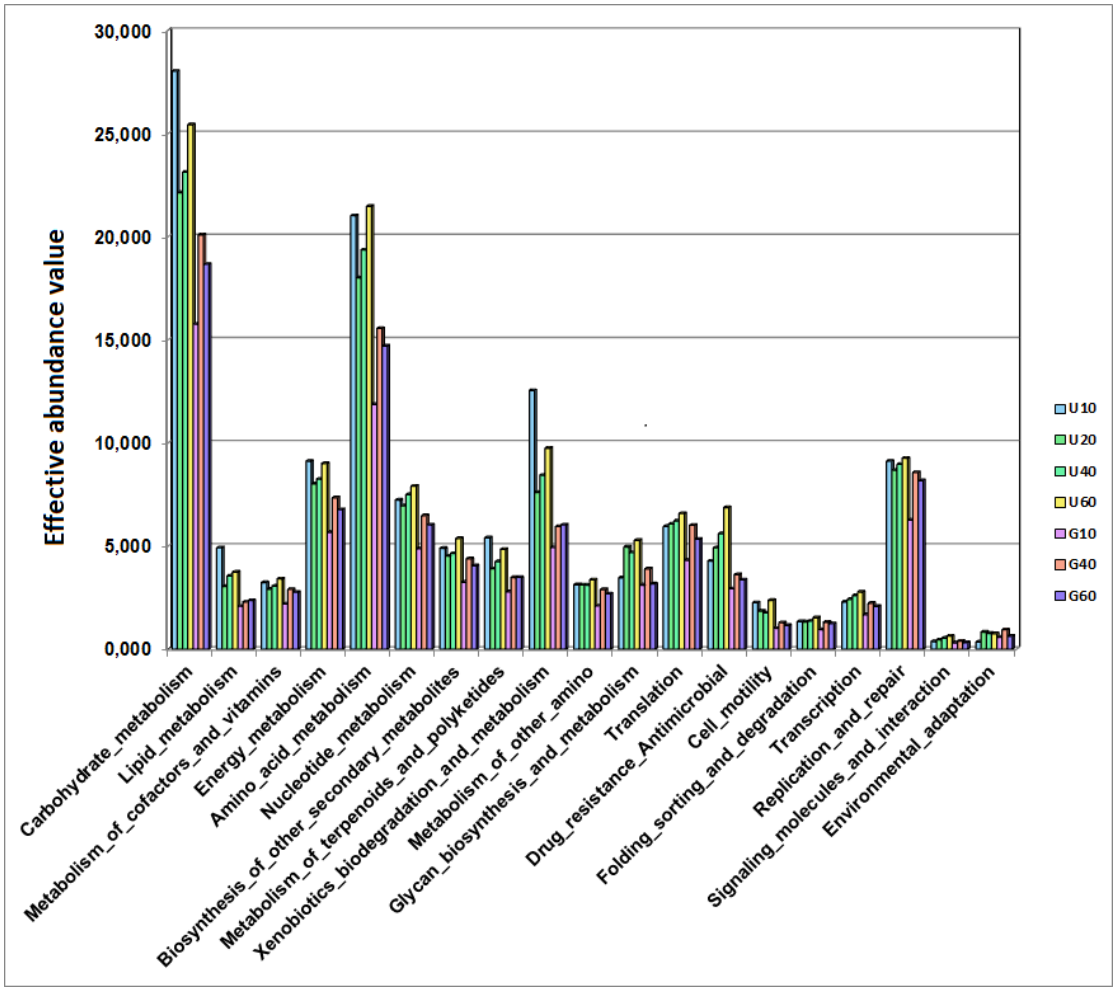

Figure A3. Functional characterization of microbial communities on PS samples using the KEGG pathway database.

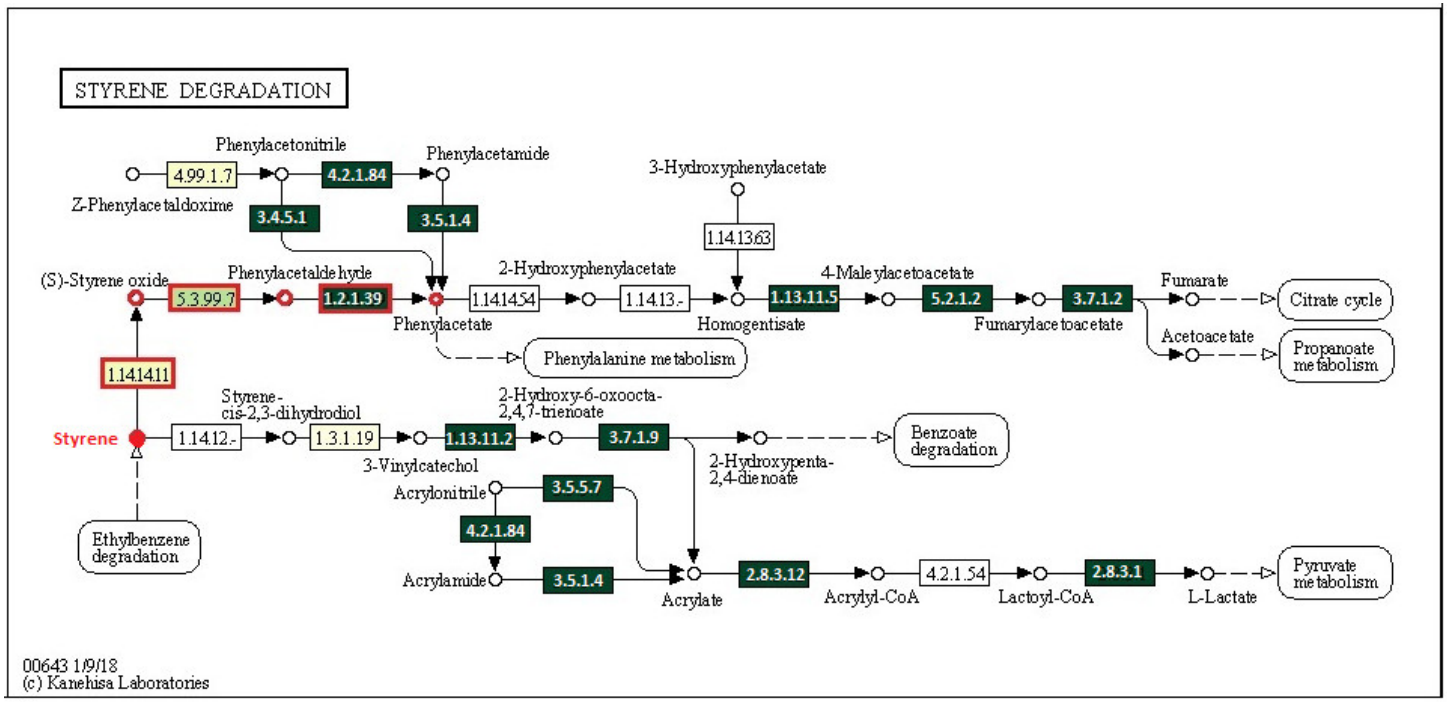

Figure A4. Predicted enzyme profile for the "Styrene degradation" pathway in all samples of the Industrial and Marine groups. The graphs were generated with the KEGG Color mapper tool (http://www.genome.jp/kegg/tool/map_pathway3.html) using the Color Mapping files generated by the Local Mapper module of iVikodak. The color intensity indicates an average gene (enzyme) proportion across all samples. The styrene degradation pathway probably employed by the biofilm bacteria is marked in red.

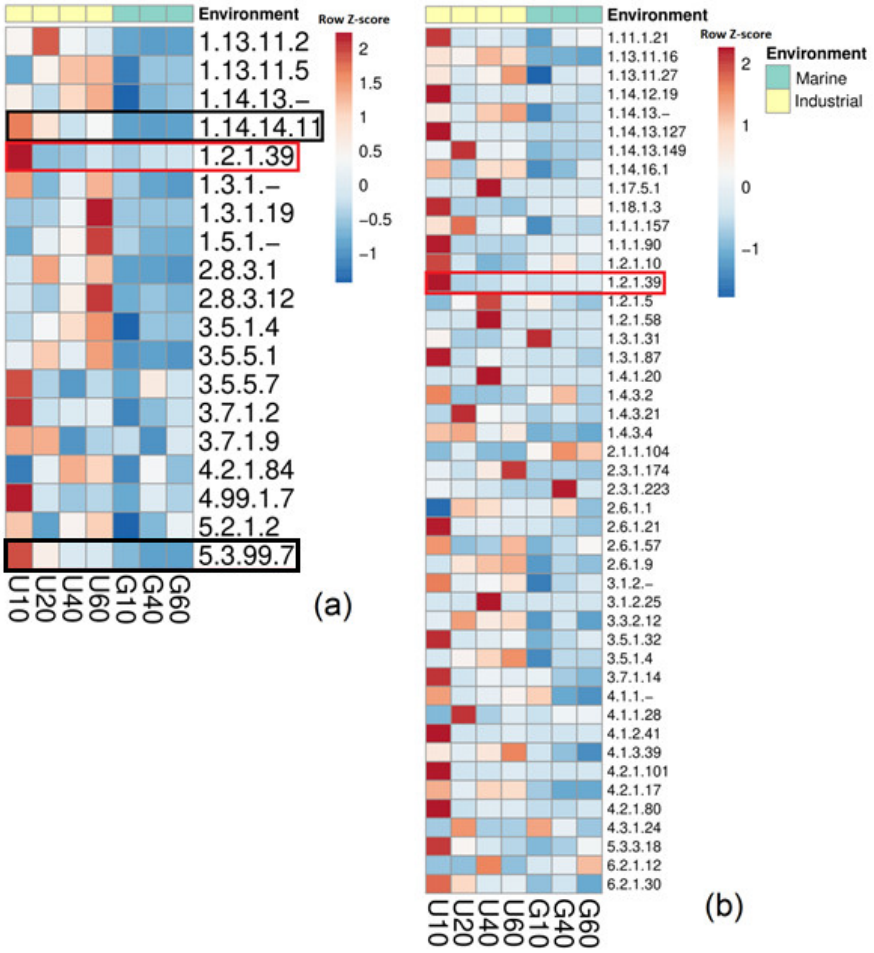

Figure A5. Heatmap showing the clustering of PS samples based on the selected iVicodak-derived enzyme profiles of the "Styrene degradation" (**a**) and "Phenylalanine metabolism" (**b**) metabolic pathways. Specific enzymes of styrene degradation are marked in black; the enzyme catalyzing formation of phenylacetic acid, the main intermediate metabolite, is marked in red.

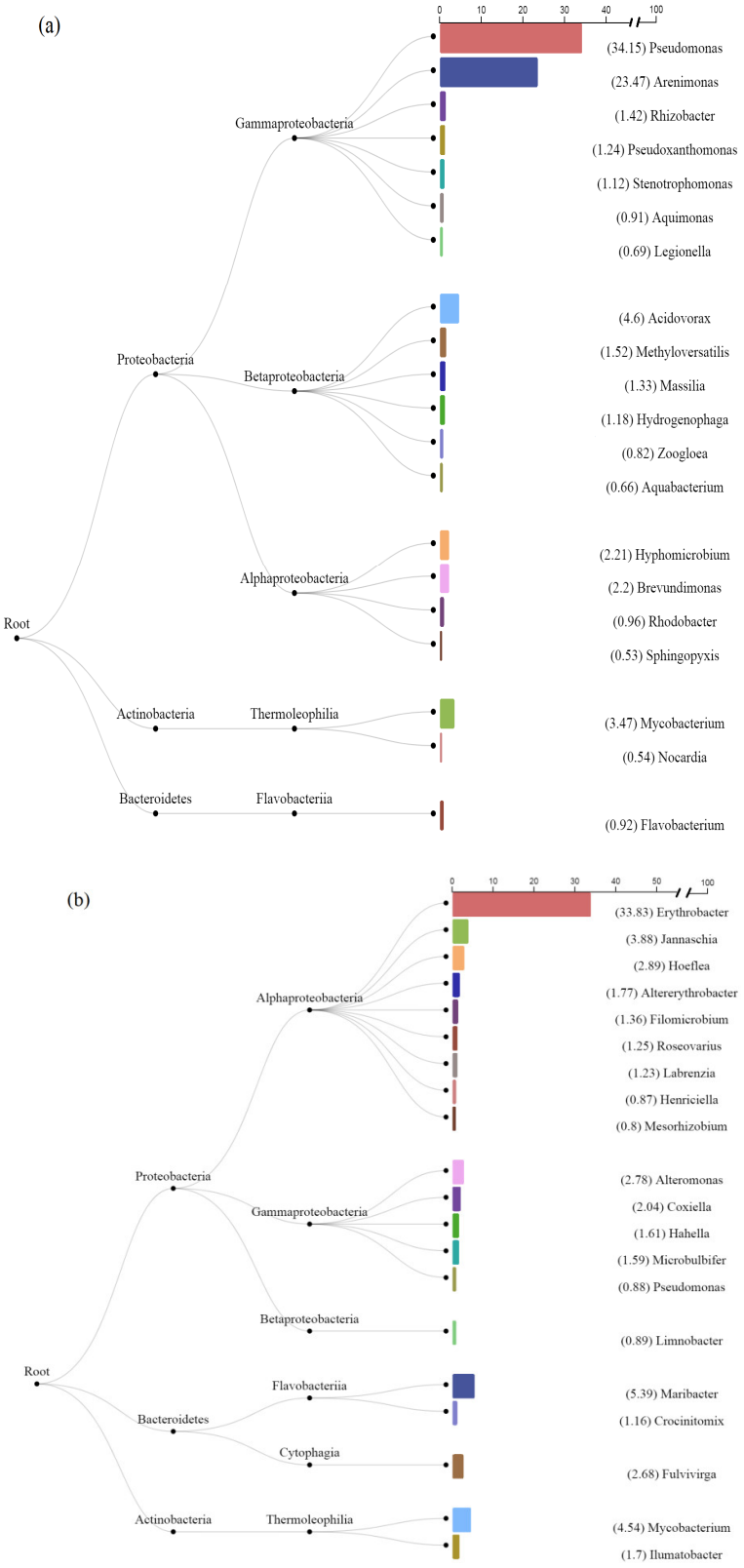

Figure A6. Dendrobar plots, highlighting the key contributing microorganisms (and their taxonomic lineage) involved in the "Styrene degradation" pathway in biofilms from PS samples incubated in industrial water (**a**) and seawater (**b**).

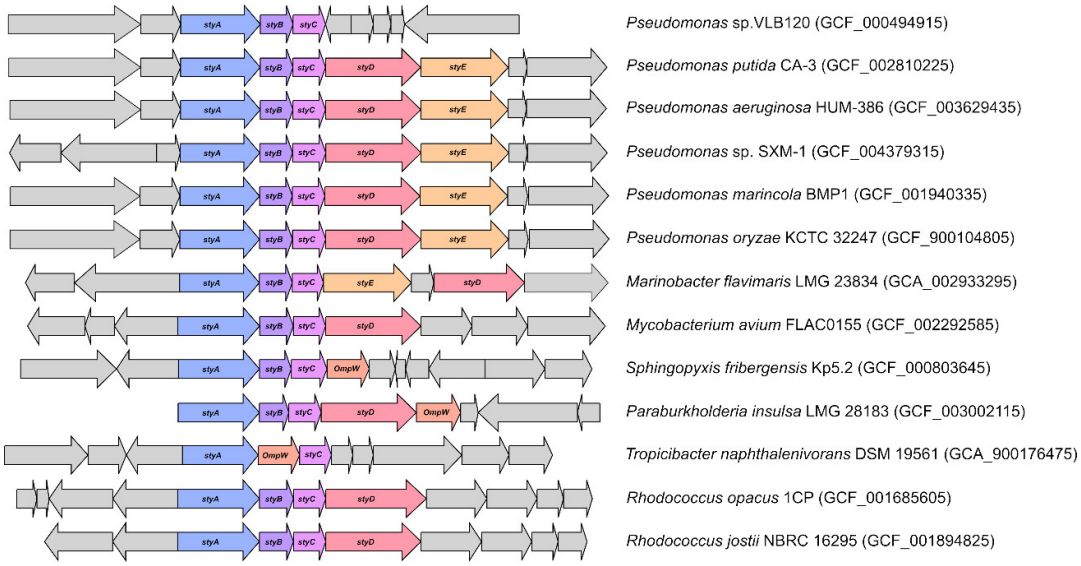

Figure A7. Comparison of the organization of the *styABCD* operon in bacterial genomes.

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