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Dominance of Endospore-forming Bacteria on a Rotating Activated Bacillus Contactor Biofilm for Advanced Wastewater Treatment

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The bacterial diversity inherent to the biofilm community structure of a modified rotating biological contactor wastewater treatment process, referred to as the Rotating Activated Bacillus Contactor (RABC) process, was characterized in this study, via both culture-dependent and culture-independent methods. On the basis of culture-dependent methods, *Bacillus* sp. were found to exist in large numbers on the biofilm (6.5% of the heterotrophic bacteria) and the microbial composition of the biofilms was quite simple. Only three phyla were identified-namely, the *Proteobacteria*, the *Actinobacteria* (High G+C Gram-positive bacteria), and the *Firmicutes* (Low G+C Gram-positive bacteria). The culture-independent partial 16S rDNA sequence analysis revealed a considerably more diverse microbial composition within the biofilms. A total of eight phyla were recovered in this case, three of which were major groups: the *Firmicutes* (43.9%), the *Proteobacteria* (28.6%), and the *Bacteroidetes* (17.6%). The remaining five phyla were minor groups: the *Planctomycetes* (4.4%), the *Chlorobi* (2.2%), the *Actinobacteria* (1.1%), the *Nitrospirae* (1.1%), and the *Verrucomicrobia* (1.1%). The two most abundant genera detected were the endospore-forming bacteria (31.8%), *Clostridium* and *Bacillus*, both of which are members of the *Firmicutes* phylum. This finding indicates that these endospore-forming bacteria successfully colonized and dominated the RABC process biofilms. Many of the colonies or clones recovered from the biofilms evidenced significantly high homology in the 16S rDNA sequences of bacteria stored in databases associated with advanced wastewater treatment capabilities, including nitrification and denitrification, phosphorus accumulation, the removal of volatile odors, and the removal of chlorohydrocarbons or heavy metals. The microbial community structures observed in the biofilms were found to correlate nicely with the enhanced performance of advanced wastewater treatment protocols.

Keywords: Rotating Activated Bacillus Contactor (RABC), biofilm, microbial community structure, endospore-forming bacteria, advanced wastewater treatment

Due to their highly efficient removal of nitrogen, phosphorus, and highly concentrated organics, and also because they eliminate undesirable odors, biological wastewater treatment processes predominantly utilizing *Bacillus* species have recently become the focus of a great deal of attention in the Republic of Korea (Kim *et al.*, 2004). Existing technologies that employ *Bacillus* species are predicated on the suspended-growth treatment process; a modified activated sludge process is one example of this technology. A new wastewater treatment technology has been developed on the basis of the attached-growth process, using a rotating biological contactor (RBC); this process employs *Bacillus* in densely flocked biofilms affixed to multiple-stage reticular rotating carriers. The *Bacillus* species function as the predominant microbial group for advanced municipal wastewater treatment; thus, the process is referred to as the Rotating Activated Bacillus Contactor (RABC) process (Kim *et al.*, 2004). However, Kim *et al.* (2004) previously analyzed the relevant microbial group only via culture-dependent methods, which were not capable of determining the actual structure.

The RABC is comprised of an attached-growth unit and a suspended-growth unit for the selection, via starvation, of endospore-forming bacteria. A suspended-growth unit is composed of a series of small aeration tanks with tapered aeration. The process has evidenced extremely high removal efficiency for inorganic nutrients, including nitrogen and phosphorus, as well as for organic materials (Murakami *et al.*, 1995; Kim *et al.*, 2004). Kim *et al.* (2004) previously

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reported that the RABC process evidenced stable high removal efficiency, yielding a BOD_5 of less than 10 mg/L, a total nitrogen content of 15 mg/L, and a total phosphorus content of 1.5 mg/L in the final effluent. Additionally, the process generates no undesirable odors in the traditional rotating biological contactor (RBC). It is believed that these effects are attributable to the activities of the *Bacillus* bacterial group. A few papers on the microbial composition of RBC biofilms have been dedicated to the treatment of nitrogen-rich wastewater (Egli *et al.*, 2003; Pynaert *et al.*, 2003). Park *et al.* (2003) and Cho *et al.* (2003) reported the purification of some exceptional enzymes that were capable of degrading organic pollutants in the wastewater from RABC biofilms. However, the microbial composition or structure of the process has yet to be determined via culture-independent methods.

Herein, we have focused on the verification of the predominance of endospore-forming bacteria, particularly *Bacillus*, within the biofilm, as well as on the identification of the bacterial structure of the RABC biofilm on the basis of both culture-dependent and culture-independent methods. The numbers of *Bacillus* and total heterotrophic bacteria within the biofilm were enumerated via the plate count technique. The 16S rDNAs from the RABC biofilms were amplified, and all clones were categorized into phylogenetic types on the basis of restriction fragment length polymorphism (RFLP). Further partial sequences of representative clones of the different phylogenetic types were aligned, and a phylogenetic tree was constructed.

Materials and Methods

Biofilm samples

Slurry biofilm samples were obtained from the running pilot-scale (capacity, 50 m^3/d) and full-scale (300 m^3/d) RABCs, which were built within the Nanji wastewater treatment plant in Seoul and within the Tongbok wastewater treatment plant in Pyeongtaek, Republic of Korea, respectively, in 2003. The RABC biofilms were formed naturally via the addition of sludge cakes acquired from a pre-existing human manure treatment plant in Ulsan, Korea, which was operated via a modified activated sludge process. RBC biofilm samples were collected from the Suanbo wastewater treatment plant (70 m^3/d) located in Chungbuk Province, Republic of Korea. The activated sludge samples were collected from the completely mixed aeration tank effluent within the Daejeon sewage treatment plant (150,000 m^3/d) located in Daejeon, Republic of Korea. All samples were transferred to the laboratory for processing within 2 h of collection.

Viable counts of Bacillus and heterotrophic bacteria

Five milliliters of slurry biofilm samples were diluted to 1:10 in sterile sodium phosphate buffer (45 ml; 0.1 M, pH 7.0) containing 0.85% (w/v) NaCl. The samples were then processed via vortex agitation (for 1 min), sonication (1 min on and 30 s off, repeated three times) (Herbert, 1990), and centrifugation (3,000×g, 2 min) for detachment from the slurry particles. The supernatants were utilized for the experiments. Bacterial cells within the supernatants of the biofilm samples were enumerated via serial dilution in phosphate-buffered saline, which was agitated vigorously for 1 min prior to plating onto *Bacillus* medium (nutrient broth 8 g/L, glucose 8 g/L, NaCl 6 g/L, agar 15 g/L) for the detection of *Bacillus* (Murakami *et al.*, 1995), and tryptic soy agar (TSA) medium (Atlas, 1997) for the detection of heterotrophic bacteria. All media were obtained from Difco and BBL (Sparks, USA). All plates were incubated for 48 h at 37°C. The numbers of typical *Bacillus* colonies on the media were counted for a presumptive colony count on the basis of typical *Bacillus* morphology; large, round colonies of a white, gray, or creamy color resulting from the generation of excessive slimy extracellular polymeric substances (EPS). The presumptive count was revised to a confirmed count on the basis of the properties of Gram-positive, rod-shaped, and endospore-forming colonies. All of the bacterial colonies grown on the TSA plates were counted as the numbers of heterotrophic colonies (Smibert and Krieg, 1994).

Identification of Bacillus and heterotrophic bacteria

Typical *Bacillus* colonies on the culture plates were isolated and pure-cultured, and the Gram-positive and endosporulated rod-shaped isolates were then identified via 16S rDNA sequence analyses. Colony PCR without DNA extraction was conducted in accordance with the protocols described in the 'PCR, cloning, and sequencing' section. Each of the purified heterotrophic bacterial isolates on TSA media were characterized via Gram staining, catalase and oxidase tests, and endospore formation. The MIDI identification system based on the fatty acid methyl ester (FAME) profiles of the cell membranes was then applied in order to identify the heterotrophic bacteria (Lee and Kim, 2003).

Collection of bacterial community and DNA extraction

For DNA extraction, 2 ml of each of the biofilm slurry samples were added to 250 mg of sterile silica beads (0.1-mm in diameter) in a microcentrifuge tube. Two ml of sterile SDS buffer [100 mM NaCl, 500 mM Tris HCl (pH 8.0), 10% SDS] was added and shaken three times for 80 seconds at maximum speed in a mini bead-beater (McBain *et al.*, 2003). The beads were permitted to settle without centrifugation, and the supernatant was transferred to a fresh 1.5 ml Eppendorf tube. The supernatant was centrifuged for 15 min at 12,000×g at 4°C and utilized for DNA extraction. The total nucleic acids were extracted using lysozymes, freeze-thawing, and phenol-chloroform, as previously described (Lee *et al.*, 1996). The extracted nucleic acids were purified via electrophoresis through a 0.75% low-melting agarose gel (SeaPlaque GTG, FMC Bioproducts, USA). The total DNAs were purified from the excised gel slices using a DNA purification kit (DNA PrepMate, Bioneer Co., Korea), eluted in 30 μl of sterile TE buffer, and used as PCR templates.

PCR, cloning, and sequencing

Bacterial 16S rDNA was amplified with the two universal primers, 27F; 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R; 5'-GGYTACCTTGTTACGACTT-3' (Lane, 1991). PCR amplification was conducted using a thermal cycler (PTC-100™, MJ Research Inc., USA) under the following conditions: initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 1.5 min, annealing at 50°C for 1

min, and extension at 72°C for 2 min, followed by a final extension step at 72°C for 30 min. The reaction mixtures (final volume, 50 μl) contained 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.8 at 25°C), 50 mM KCl, 0.1% Triton X-100, 200 ng of bovine serum albumin per μl, 200 μM each deoxynucleoside triphosphate (dNTPs), 0.2 μM each oligonucleotide primer, and 2 U of Dynazyme (Finnzymes Oy., Finland). Approximately 50 ng of DNA template was added to each of the PCR tubes. The amplification products were pooled, and the bands corresponding to the correctly sized product (1.5 kb) were excised, purified, and concentrated. The 16S rDNA library was generated using the purified 16S rDNAs from the samples via ligation into pGEM-T vector (Promega, USA). Ligation, transformation into *E. coli* DH5α-competent cells, and blue/ white screening were conducted in accordance with the manufacturer's instructions. Plasmid inserts were amplified directly from the transformant cells via colony PCR with the T7 and SP6 pGEM-T primers for 16S rDNA screening. The composition of the reaction mixtures and PCR coditions were as described above. The diversity of the clone library was verified via RFLP. Aliquots (5.0 μl) of the reamplified PCR products were digested for 3 h at 37°C with 2 U of *Hae*III (Promega, USA). The resultant fragments were separated via gel electrophoresis in 4% NuSieve 3:1 agarose (FMC Bioproducts, USA). The clones were ordered in different RFLP patterns, and representative clones of each were selected and sequenced. The plasmid preparations for DNA sequencing were created using Wizard Mini-Preps (Promega, USA). The rDNA sequencing was conducted using the Automated DNA Sequencer (ABI PRISM™ 37, Perkin Elmer, USA).

Phylogenetic analyses

Analyses and homology searches of the 16S rDNA sequences of approximately 550 bases were completed using sequences available in the BLAST network service (blastn) of the National Center for Biotechnology and Information (NCBI, http://www.ncbi.nlm.nih.gov/BLAST) in order to evaluate their phylogenetic affiliations. The sequences were initially aligned using the CLUSTAL X (1.81) program (Thompson *et al.*, 1997), examined visually, then relocated to allow for maximal alignment by referring to representative bacterial sequences obtained from the NCBI database. Sequences were also checked for chimeric properties using the Chimera Check program from the RDP Database Project II (http://rdp8.cme.msu.edu/html/analyses.html). Phylogenetic trees were constructed via the Bootstrap Neighbor-Joining Tree option in the CLUSTAL X (1.81) program. *Pseudomonas putida* of the phylum *Proteobacteria* was used as the outgroup for the *Bacillus* isolates, and *Methanobacterium thermoflexum* of the *Archaea* domain was utilized as the outgroup for the 16S rDNA clones. The bootstrap values were shown as percentages of 100 replications. The tree was viewed using the TreeView program (Win32) 1.5.2 (http://taxonomy.zoology.gla.ac.uk/rod/rod.html) (Page, 1996).

Nucleotide sequence accession number

The partial sequences revealed in this study were deposited in the GenBank database under the accession numbers AF539642-AF539696 for the *Bacillus* isolates, designated by the prefix BfNi- in Fig. 1, and DQ106922-DQ106984 for the bacterial clones, designated by the prefix Bf- in Fig. 2.

Results and Discussion

Dominance of Bacillus from culture-dependent analyses

Table 1 shows that the number of culturable *Bacillus* grown on the Bacillus medium was between 6 and 7 logs per milliliter, an average of 6.5% of the number of heterotrophic bacteria on TSA medium under oxic culture conditions. This value was substantially higher than in the RBC biofilm (5 logs, 0.4%) or activated sludge (4 logs, 1.4%) (Table 1). Bitton (1999) previously reported that the average distribution of *Bacillus* in standard activated sludge processes comprised 1.9% of the aerobic heterotrophic bacteria. The higher proportion of *Bacillus* appears to be quite important in terms of RABC functions including biomass and activity. Ellis *et al.* (2003) emphasized that the readily culturable component of microbial communities might be the most relevant in terms of function, and that positive correlations could be drawn between activity and cell size, between cell size and culturability, and between activity and culturability.

Whole colonies on the TSA medium plates were pure-cultured and identified using the MIDI system. All isolates with a confidence level of less than 0.450 were considered to be unclassifiable. As is shown in Table 2, the RABC biofilms harbored two major groups, the *Proteobacteria* (γ- and β-, 35.8%) and the *Actinobacteria* (34.0%). The collection of isolated colonies did not harbor α- or δ-*Proteobacteria*, probably due to the fact that members of these groups are

Table 1. Numbers of *Bacillus* and heterotrophic bacteria (CFU/ml) and ratio of *Bacillus* (%) in various wastewater treatment processes including RABC, RBC, and completely-mixed activated sludge

Name of wastewater treatment plant	Process type	Sample	No. of *Bacillus* (A)	No. of heterotrophic bacteria (B)	% of *Bacillus* (A/B)
Pilot-scale RABC[a]	RABC	biofilm	2.4×10^6	3.6×10^7	6.7%
Full-scale RABC[b]	RABC	biofilm	1.2×10^7	1.9×10^8	6.3%
Suanbo WWTP[c]	RBC[e]	biofilm	1.0×10^5	2.4×10^7	0.4%
Daejeon City STP[d]	activated sludge	aeration tank effluent	1.5×10^4	1.1×10^6	1.4%

[a,b] Rotating activated bacillus contactor process, treatment capacity of 50 and 300 m^3/d, respectively.
[c] Wastewater treatment plant, treatment capacity of 70 m^3/d.
[d] Sewage treatment plant, treatment capacity of 150,000 m^3/d.
[e] Rotating biological contactor process.

Table 2. Composition of aerobic or facultatively anaerobic heterotrophic bacteria in biofilms of the RABC process based on culture-dependent methods. Identification was performed by fatty-acid-methyl-ester analysis.

Phylogenetic division	No. of isolates	% of total	Mean confidence level (range)
Total	53	100.0	
Proteobacteria	19	35.8	
γ-*Proteobacteria*	17	32.0	
Aeromonas	11	20.7	0.755(0.636~0.898)
Stenotrophomonas	4	7.5	0.856(0.843~0.868)
Acinetobacter	1	1.9	0.878
Pseudomonas	1	1.9	0.816
β-*Proteobacteria*	2	3.8	
Acidovorax	2	3.8	0.617(0.498~0.736)
Actinobacteria	18	34.0	
Corynebacterium	5	9.5	0.601(0.483~0.755)
Arthrobacter	4	7.5	0.655(0.510~0.825)
Rhodococcus	4	7.5	0.524(0.473~0.583)
Microbacterium	2	3.8	0.513(0.459~0.566)
Tsukamurella	1	1.9	0.578
Gordona	1	1.9	0.784
Kocuria	1	1.9	0.910
Firmicutes	1	1.9	
Bacillus (Paenibacillus)	1	1.9	0.785
Unclassifiable	15	28.3	0.141(0~0.340)

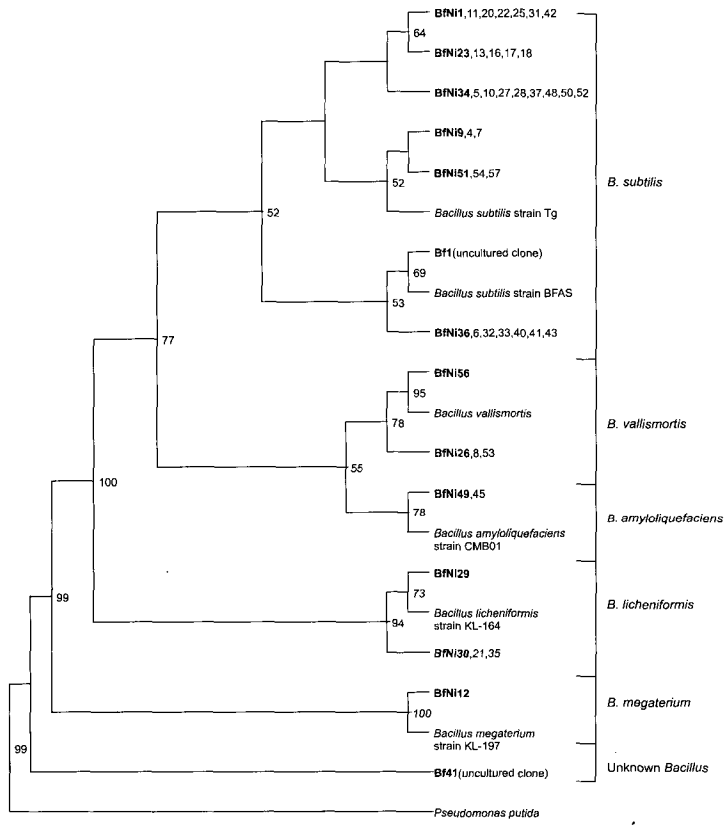

Fig. 1. Rectangular cladogram of *Bacillus* isolates or clones from the biofilms on the basis of the analysis of ca. 550 bases of aligned 16S rDNA sequences. Bootstrap values (>50%) generated from 100 replicates are shown at each node. Sequences of the isolates or clones obtained from the samples are designated by the prefix BfNi or Bf, followed by replicated numbers (BfNi1, Bf1, etc.). All sequences of the isolates and clones were deposited into the GenBank database. The boldfaced numbers are those for which sequences are used for the tree. *Pseudomonas putida* in the γ-*Proteobacteria* phylum was utilized as the outgroup.

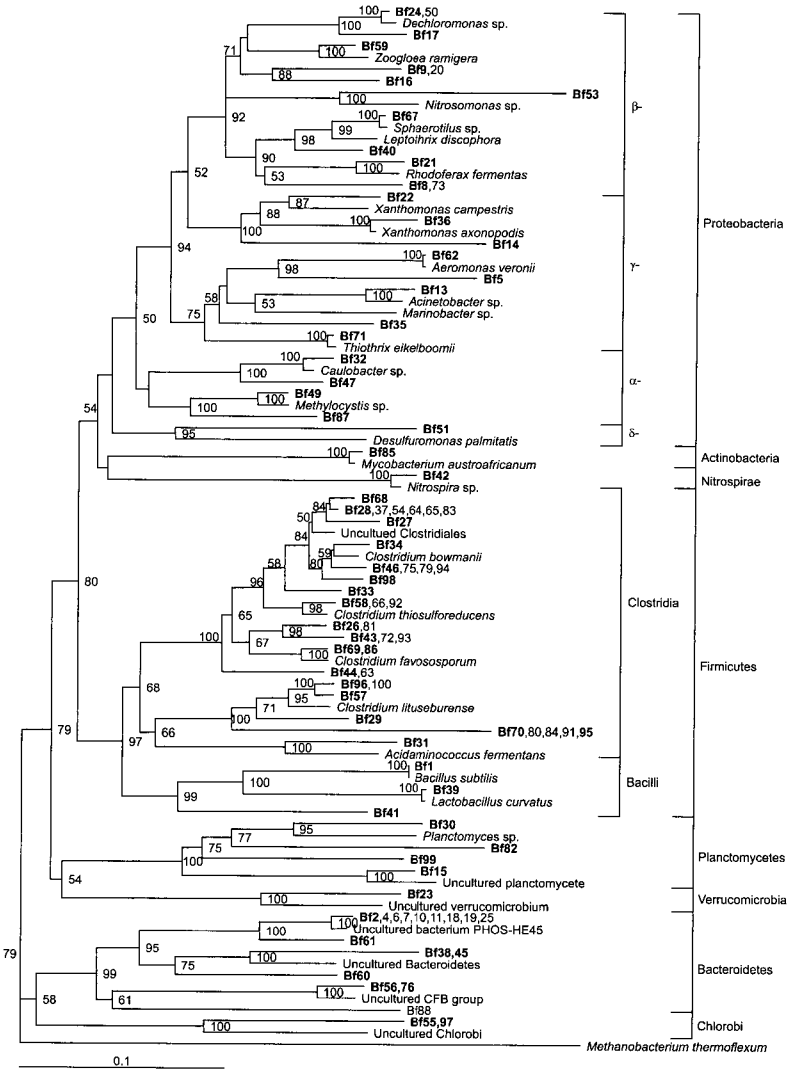

Fig. 2. Phylogenetic tree generated via the neighbor-joining method showing the phylogenetic relationships of the RABC biofilm clones within the Domain *Bacteria* based on the analysis of ca. 550 bases of aligned 16S rDNA sequences. Bootstrap values (>50%) generated from 100 replicates are shown at each node. Sequences obtained from samples are designated in boldface by the prefix Bf, followed by replicated numbers (Bf1, Bf2, Bf3, etc.). The boldfaced clone numbers were deposited into the GenBank database. *Methanobacterium thermoflexum* in the Domain *Archaea* was utilized as the outgroup. The scale bar represents 0.1 change per nucleotide.

generally slow-growing or may have specific physiological requirements, and therefore may have been lost during the subculturing process (Ellis *et al.*, 2003). Only one colony of *Firmicutes* was detected on the plate. The others (28.3%) were unidentifiable. The TSA medium does not appear to be appropriate for the isolation of *Bacillus,* as it allows for the rapid growth of eutrophic bacteria, such as the *Proteobacteria* or the *Actinobacteria*. Conversely, *Bacillus* species were recovered efficiently from the *Bacillus* medium.

Identification of Bacillus colonies by 16S rDNA sequences

A total of 55 presumptive *Bacillus* colonies were selected on the basis of colony morphology, and were further identified by their 16S rDNA sequences, 49 of which proved to be *Bacillus*, with an 89% recovery rate (Fig. 1). As the 16S rDNA sequences of all of the *Bacillus* isolates were so similar, the phylogenetic tree was difficult to figure out in phylogram mode. Hence, we constructed the tree in rectangular cladogram mode (Fig. 1).

A total of six *Bacillus* species were identified from the biofilm (Fig. 1). Five of these species were recognized as members of Group II or the *Bacillus subtilis* group, which are capable of fermenting a variety of sugars, including glucose, and of growing slowly in the presence of nitrate under anoxic conditions (Priest, 1993). The remaining species was classified as an unknown *Bacillus*. The predominant species was *B. subtilis*, and the other four were *B. vallismortis*, *B. amyloliquefaciens*, *B. licheniformis,* and *B. megaterim*. Kim *et al.* (2004) reported that the RABC process evidenced a removal rate of approximately 50% in total nitrogen, and maintained less than 15 mg/L of total nitrogen concentration in the final effluent with the same sample of this

Table 3. Composition of bacterial groups in biofilms of the RABC process based on culture-independent 16S rDNA analyses

Phylogenetic division	No. of clones	% of total
Total	91	100.0
Firmicutes	40	43.9
Clostridia	37	40.6
Clostridium	28	30.7
Uncultured clostridia	8	8.8
Acidaminococcus	1	1.1
Bacilli	3	3.3
Bacillus	1	1.1
Uncultured bacilli	1	1.1
Lactobacillus	1	1.1
Proteobacteria	26	28.6
β-Proteobacteria	13	14.3
Zoogloea	4	4.4
Dechloromonas	3	3.3
Derxia	2	2.2
Leptothrix	1	1.1
Rhodoferax	1	1.1
Sphaerotilus	1	1.1
Nitrosomonas	1	1.1
γ-Proteobacteria	8	8.8
Xanthomonas	2	2.2
Pseudoxanthomonas	1	1.1
Aeromonas	1	1.1
Acinetobacter	1	1.1
Enterobacter	1	1.1
Thiothrix	1	1.1
Marinobacter	1	1.1
α-Proteobacteria	4	4.4
Caulobacter	2	2.2
Uncultured Azospirillum	1	1.1
Methylocystis	1	1.1
δ-Proteobacteria	1	1.1
Desulfuromonas	1	1.1
Bacteroidetes	16	17.6
Uncultured bacterium	10	11.0
Uncultured Bacteroidetes	3	3.3
Uncultured CFB group	2	2.2
Flexibacter	1	1.1
Planctomycetes	4	4.4
Uncultured planctomycete	2	2.2
Planctomyces	1	1.1
Uncultured bacterium	1	1.1
Chlorobi	2	2.2
Uncultured Chlorobi bacterium	2	2.2
Actinobacteria	1	1.1
Mycobacterium	1	1.1
Nitrospirae	1	1.1
Nitrospira	1	1.1
Verrucomicrobia	1	1.1
Uncultured verrucomicrobium	1	1.1

study. The nitrate respiration observed in this group appears to contribute to the excellent nitrogen removal efficiency associated with the RABC process.

Composition of the RABC biofilm based on culture-independent analyses

A total of 95 16S rDNA clones were obtained from the DNA extraction without culture. Four clones appeared to be chimeras, and were thus excluded from the phylogenetic analyses. The phylogenetic assignment of the remaining 91 clones to bacterial divisions was conducted using the EMBL/GenBank database in the BLAST network service; phylogenetic affiliations are provided in Fig. 2. 31 of 91 sequences (34.1%) evidenced similarities of greater than 97.0% with the sequences obtained from the database, indicating the potentially identical clones. Ellis *et al.* (2003) reported that the coverage was 30.7%, with a sequence similarity of >97%, in the heavy metal-contaminated soils. 32 sequences (35.1%) evidenced similarity values between 97% and 90%, and 28 sequences (30.8%) evidenced similarity values lower than 90.0%. A total of eight different phyla were identified. 90% of the clones were assigned to three well-characterized divisions, the *Firmicutes* (43.0%), the *Proteobacteria* (28.6%), and the *Bacteroidetes* (17.6%). The five minor groups were as follows: the *Planctomycetes*, the *Chlorobi*, the *Actinobacteria*, the *Nitrospirae*, and the *Verrucomicrobia*. Table 3 shows significant differences in the microbial compositions of the RABC biofilms as compared to the culture-dependent FAME results provided in Table 2.

Dominance of endospore-forming bacteria in the RABC biofilms

The results of culture-independent molecular analyses (Table 3) indicated that the numbers of *Bacilli* and *Clostridia* endosporulating bacterial clones constituted 43.9% of the total, thereby suggesting that the RABC biofilms were dominated by these endosporulating bacteria. It is clear that the RABC biofilms harbored a great deal of endospore-forming bacteria as compared to other wastewater treatment systems, as *Bacillus* occupied between 6.3 and 6.7% of the total HPC bacteria (Table 1) and endosporulating *Firmicutes* was most dominant (43.9%) according to the culture-independent method (Table 3). The strictly anaerobic endospore-forming Gram-positive bacteria, *Clostridia*, were the principal group (41.7%) detected within the biofilm of this aerobic wastewater treatment plant, and *Bacillus* clones constituted only 2.2% (Table 3). We proposed that the dominance of the endospore-formers resulted from starvation within the system, in which the RABC effluent was treated in a series of suspended-growth biological aeration tanks. The BOD_5 concentration in the effluent of the last suspended-growth biological reactor was reduced to 6 mg/L from 140 mg/L in the wastewater coming into the RABC reactor (Kim *et al.*, 2004). We confirmed, via microscopy, that the effluent harbored large numbers of sporulated cells. This implies that the bacterial groups in the RABC biofilms that were most relevant to wastewater treatment were *Bacilli* in the oxic compartments and *Clostridia* in the anoxic compartments. The over-abundance of strictly anaerobic *Clostridia* bacteria, rather than *Bacilli*, when using the culture-independent method appears to be the result of a

Table 4. Sequence identities and characteristics of the important biofilm bacterial clones that appear in Fig. 2 based on nucleotide-nucleotide BLAST (blastn) of the National Center for Biotechnology Information (NCBI).

Clone	Accession number	Best match in NCBI database	Group level affiliation	Similarity (%)	Source or characteristics of the best match
Bf1	DQ106922	*Bacillus* sp. Ni-19	*Firmicutes: Bacilli*	99	Rotating biological contactor biofilm
Bf2	DQ106923	Uncultured bacterium PHOS-HE45	*Bacteroidetes*	98	Phosphorus-removal ecosystem and nitrate respiration
Bf5	DQ106924	Uncultured bacterium clone A9	*γ-Proteobacteria*	95	Reductive dechlorination
Bf8	DQ106925	Uncultured bacterium clone 3	*β-Proteobacteria*	95	Denitrifying reactor
Bf9	DQ106926	Uncultured bacterium clone UTFS-OF08-d36-08	*β-Proteobacteria*	99	EBPR[b] activated sludge
Bf15	DQ106929	Uncultured planctomycete clone CYOARA055E08	*Planctomycetes*	96	Municipal WWTP[a]
Bf17	DQ106931	Uncultured betaproteobacterium clone UCT N123	*β-Proteobacteria*	95	Phosphorus-removal WWTP
Bf21	DQ106932	Uncultured bacterium clone FB302-A6	*β-Proteobacteria*	97	Subsurface sediment contaminated with uranium and nitrate
Bf24	DQ106935	Uncultured bacterium clone UTFS-OF08-18	*β-Proteobacteria*	98	EBPR activated sludge
Bf27	DQ106937	*Clostridium tunisiense*	*Firmicutes: Clostridia*	94	Proteolyitc, sulfur-reducing anaerobe
Bf28	DQ106938	*Clostridium tunisiense*	*Firmicutes: Clostridia*	96	Proteolyitc, sulfur-reducing anaerobe
Bf30	DQ106940	Unidentified organism	*Planctomycetes*	98	Phosphate-removing activated sludges
Bf31	DQ106941	Uncultured bacterium clone ER1_17	*Firmicutes: Clostridia*	93	Anaerobic biological WWTP
Bf35	DQ106945	*Marinobacter* sp. SBS	*γ-Proteobacteria*	90	Anaerobic respiration utilizing humic substances
Bf36	DQ106946	*Xanthomonas axonopodis* strain S53	*γ-Proteobacteria*	97	Activated sludge
Bf38	DQ106947	Uncultured bacterium clone DSBR-B050	*Bacteroidetes*	94	Denitrifying microbes
Bf40	DQ106949	*Leptothrix* sp. L14	*β-Proteobacteria*	95	Filamentous bacteria in the sludge bulking
Bf41	DQ106950	Uncultured soil bacterium clone TcA34	*Firmicutes: Clostridia*	97	Heavy metal-contaminated bulk soil
Bf42	DQ106951	*Nitrospira* sp. strain RC7	*Nitrospirae: Nitrospira*	97	Nitrite-oxidizing bioreactor
Bf44	DQ106953	Uncultured soil bacterium clone TcA10	*Firmicutes: Clostridia*	98	Heavy metal-contaminated bulk soil
Bf45	DQ106954	Uncultured bacterium clone DSBR-B050	*Bacteroidetes*	98	Denitrifying microbes
Bf49	DQ106957	*Methylocystis* sp. KS7	*α-Proteobacteria*	96	Methane-oxidizing
Bf51	DQ106958	Uncultured deltaproteobacterium clone JG37-AG-55	*δ- Proteobacteria*	88	Uranium mining waste piles
Bf53	DQ106959	*Nitrosomonas* sp. Nm84	*β-Proteobacteria*	89	Ammonia-oxidizing
Bf55	DQ106960	Uncultured eubacterium clone F13.30	*Chlorobi*	92	Toxic and refractory WWTP
Bf56	DQ106961	Uncultured bacterium clone BIOEST-2	*Bacteroidetes*	96	Oxidation of sulfide-containing wastewater
Bf57	DQ106962	Uncultured bacterium clone SHA-123	*Firmicutes: Clostridia*	96	Dechlorination of dichlorpropane
Bf58	DQ106963	Uncultured bacterium clone 053H08_B_DI_P58	*Firmicutes: Clostridia*	98	Municipal anaerobic sludge digester
Bf60	DQ106965	Uncultured bacterium MK21	*Bacteroidetes*	88	Activated sludge
Bf61	DQ106966	Uncultured Bacteroidetes bacterium clone Bihii16	*Bacteroidetes*	96	Waste gas-degrading biofilter
Bf67	DQ106968	*Sphaerotilus* sp. L19	*β-Proteobacteria*	99	Filamentous bacteria in the sludge bulking
Bf68	DQ106969	Metal-contaminated soil clone K20-13	*Firmicutes: Clostridia*	95	Metal-contaminated site
Bf71	DQ106972	Uncultured bacterium clone UTFS-OF06-d151-26	*γ-Proteobacteria*	98	EBPR[b] activated sludge
Bf76	DQ106974	Uncultured bacterium clone BIOEST-2	*Bacteroidetes*	95	Oxidation of sulfide-containing wastewater

Clone	Accession number	Best match in NCBI database	Group level affiliation	Similarity (%)	Source or characteristics of the best match
Bf82	DQ106975	Uncultured bacterium clone MIZ48	*Planctomycetes*	94	Reduction and oxidation of iron
Bf85	DQ106976	*Mycobacterium austroafricanum* isolate VMO573	*Actinobacteria*	99	PAH-degrading
Bf86	DQ106977	Uncultured anaerobic bacterium clone A-3N	*Firmicutes: Clostridia*	94	Anaerobic swine lagoons
Bf87	DQ106978	Uncultured *Azospirillum* sp. clone GCPF39	*α-Proteobacteria*	98	Nutrient-limited cave environment
Bf88	DQ106979	Uncultured bacterium PHOS-HE99	*Bacteroidetes*	93	Phosphorus-removal ecosystem and nitrate respiration
Bf97	DQ106982	Uncultured eubacterium clone F13.9	*Chlorobi*	97	Toxic and refractory WWTP

[a] Wastewater treatment plant
[b] Enhanced biological phosphorus removal system

very low dissolved oxygen (DO) concentration within the RABC bioreactor, less than 0.5 mg/L (unpublished data). As the DO was so low and oxygen could not diffuse from the surface to the depths, it appeared highly possible that the aerobic portion may have been confined only to the top layer of the biofilm, whereas an anoxic condition was maintained throughout the biofilm. Egli *et al.* (2003) reported that the typical thickness of a rotating biological contactor biofilm was up to 750 μm, and oxygen could not penetrate much further than 100-200 μm below the surface. This would help to explain the growth of both aerobic and anaerobic bacteria on the same RBC biofilm. Our results were partially consistent with those of Egli *et al.* (2003), who reported that aerobic bacteria were detected via culturing, whereas anaerobic bacteria were determined to have dominated by the culture-independent method. Some reports (Stackebrandt *et al.*, 1993; Kaiser *et al.*, 2001; Sandaa *et al.*, 2001; Ellis *et al.*, 2003) noted the extreme difficulty inherent to the detection of *Bacillus* in clone libraries, despite their presence on the plates. Several reasons have been put forward to explain this, including PCR bias, the difficulty inherent to the lysing of spores, and the ease with which these bacteria are grown on plates. Our results indicated that the distribution of endospore-forming *Bacillus* was 2.2% in the culture-independent method (Table 3). This ratio does not appear unreasonably low, considering the quantity of anaerobic bacteria found in the biofilms. The distribution of anaerobic bacteria in the RABC biofilms was estimated at approximately 50%, on the basis of the data provided in Table 3. Therefore, it was estimated that the theoretical distribution of *Bacillus* within the RABC biofilms was approximately 3%, which is basically consistent with the 2.2% observed in the clone libraries in this study.

The majority of the sequences affiliated with the *Proteobacteria* were closely associated with the cultured bacteria with a high bootstrap value. However, for the five sequences (Bf-9, 20, 16, 8, and 73) assigned to the β-*Proteobacteria*, their nearest cultured neighbors were not detected. There were a host of uncultured sequences of the *Firmicutes* within the *Clostridia* group, and almost all of the sequences assigned to the minor groups were associated with those of uncultured bacteria (Table 3, Fig. 2). In the β-*Proteobacteria* or the *Bacteroidetes*, sequences associated with nitrogen or phosphorus removal were detected (Bf-2, 8, 17, 30, 38, 42, 45 etc., see Table 4). A sequence related to the ammonium oxidizers (the *Nitrospirae*) was also recognized (Bf-42). Additionally, sequences associated with deodorization via reductive dechlorination, sulfide oxidation, or gas degradation were detected in the *Bacteroidetes*, the *Clostridia*, or the γ-*Proteobacteria* (Bf-5, 56, 57, 61, 76). These sequences may correlate quite nicely with enhanced advanced wastewater treatment performance. However, further studies will be required in order to demonstrate the functional roles of the endospore-forming bacteria, *Clostridia* and *Bacilli*, in the removal of odors, as well as organic and inorganic pollutants.

In summary, the following conclusions were drawn from the RABC biofilm by HPC, the identification of colonies by FAME and PCR, and the culture-independent method:

1. *Bacillus* existed at levels of between 6- and 7-logs CFU per milliliters of biofilm sample on *Bacillus*-selective media, and represented between 6.3 and 6.7% of the total HPC bacteria.

2. The FAME analysis of colonies detected 3 phyla, encompassing 13 genera. The *Proteobacteria* (5 genera, 35.8%) and the *Acinetobacter* (7 genera, 34.0%) were dominant, and the *Firmicutes* (1 genus, *Bacillus*, 1.9%) was minor.

3. However, the culture-independent method, direct DNA extraction, and 16S rDNA amplification identified 8 phyla and more than 30 genera, among which the *Firmicutes* predominated (43.9%).

4. Many of the clones evidenced significant homology with the reported sequences of bacteria evidencing activity consistent with nitrification and denitrification, phosphorus accumulation, the removal of volatile odors, and the removal of chlorohydrocarbons or heavy metals. However, further studies will be required to adequately corroborate these findings.

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