Stable Coexistence of Five Bacterial Strains as a Cellulose-Degrading Community

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A cellulose-degrading defined mixed culture (designated SF356) consisting of five bacterial strains (*Clostridium straminisolvans* CSK1, *Clostridium* sp. strain FG4, *Pseudoxanthomonas* sp. strain M1-3, *Brevibacillus* sp. strain M1-5, and *Bordetella* sp. strain M1-6) exhibited both functional and structural stability; namely, no change in cellulose-degrading efficiency was observed, and all members stably coexisted through 20 subcultures. In order to investigate the mechanisms responsible for the observed stability, “knockout communities” in which one of the members was eliminated from SF356 were constructed. The dynamics of the community structure and the cellulose degradation profiles of these mixed cultures were determined in order to evaluate the roles played by each eliminated member in situ and its impact on the other members of the community. Integration of each result gave the following estimates of the bacterial relationships. Synergistic relationships between an anaerobic cellulolytic bacterium (*C. straminisolvans* CSK1) and two strains of aerobic bacteria (*Pseudoxanthomonas* sp. strain M1-3 and *Brevibacillus* sp. strain M1-5) were observed; the aerobes introduced anaerobic conditions, and *C. straminisolvans* CSK1 supplied metabolites (acetate and glucose). In addition, there were negative relationships, such as the inhibition of cellulose degradation by producing excess amounts of acetic acid by *Clostridium* sp. strain FG4, and growth suppression of *Bordetella* sp. strain M1-6 by *Brevibacillus* sp. strain M1-5. The balance of the various types of relationships (both positive and negative) is thus considered to be essential for the stable coexistence of the members of this mixed culture.

In various natural and engineered environments, many species of microorganisms stably coexist by interacting with each other and effectively exert various functions. Particularly in engineered environments, achieving the functional and structural stability of microbial communities for long-term applications and various perturbations is considered to be an important issue (3, 4, 11, 12, 16, 29). However, the mechanisms responsible for the stable coexistence of many species of microorganisms have not yet been clarified. One factor hampering the elucidations of these mechanisms is the difficulty of defining all members included in such complex microflora. Furthermore, it is even more difficult to clarify the roles of each member and the relationships among each of the members of such a community. Hence, we expected that it would facilitate our understanding of these mechanisms to reproduce the stability and the function of a microflora by constructing a defined mixed culture consisting of microorganisms isolated from the microflora. Such approaches have often been applied to examine various microbial communities, especially in the field of pollutant biodegradation (2, 5, 6, 10, 31) and in biological investigations of oral bacterial communities (13, 26). In addition, some reports have described the construction of a stable coculture consisting of two bacterial strains in mutualistic or commensalistic association (10, 31). However, other reports

have noted that the addition of a third microorganism alters the relationships between two microorganisms (e.g., see references 8 and 22). In order to gain a better understanding of natural (or engineered) complex microbial communities, relationships among many (more than two) species of microorganisms should be investigated. There appears to have been no reports to date regarding the construction of a defined mixed culture composed of more than two microorganisms that was able to stably coexist for a long period of time.

If such a defined mixed culture were to be successfully constructed, it would facilitate the examination of general characteristics of all of the members in pure culture and the monitoring of the dynamics of the members in the community throughout a given cultivation period. Furthermore, by constructing a “knockout community” in which one of the members is eliminated from the defined mixed culture, the roles played by the eliminated member in situ and its impact on the other members of the community could be evaluated. This approach is derived from the same perspective as gene disruption studies, in which the role of a gene in an organism can be evaluated by the elimination of that gene. In the field of microbial ecology, similar approaches have been applied frequently to investigate the function of a specific group of microorganisms via the inhibition of a specific type of metabolism, e.g., the inhibition of methanogenic archaea with 2-bromoethanesulfonate (e.g., see reference 14) and the inhibition of sulfate-reducing bacteria with molybdate (e.g., see reference 25). However, there appears to have been no report to date regarding the specific inhibition of one of the species in a complex microbial community.

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In our laboratory, a stable microflora (designated as "original microflora") capable of effectively degrading various cellulosic materials (e.g., filter paper, cotton, and rice straw) under aerobic static conditions was constructed by a succession of enrichment cultures, as reported previously by Haruta et al. (15). The original microflora exhibited functional and structural stability; the cellulose-degrading efficiency and the composition of bacteria did not change after more than 20 subcultures. Thus far, an anaerobic cellulolytic bacterium, *Clostridium straminisolvans* CSK1 (21), and five groups of aerobic noncellulolytic bacteria (20) have been successfully isolated from the original microflora. Cellulose degradation did not occur in the pure culture of *C. straminisolvans* CSK1 under the conditions used for the original microflora (i.e., aerobic static conditions), and the efficiency of degradation was significantly low even under anaerobic conditions. On the other hand, effective cellulose degradation under aerobic conditions occurred in a coculture of *C. straminisolvans* CSK1 with one of three aerobic isolates (*Pseudoxanthomonas* sp. strain M1-3, *Brevibacillus* sp. strain M1-5, or *Bordetella* sp. strain M1-6). A four-strain mixed culture (namely, CSK+M356, also referred to as Δ FG4 in this paper) consisting of *C. straminisolvans* CSK1 and the three strains of the aerobic isolates was also found to effectively degrade cellulose. Although CSK+M356 exhibited stability for subculture with respect to function (i.e., cellulose degradation efficiency), it was not stable in terms of its structure; one of the members disappeared during subculture (20). One conceivable reason for this finding was that CSK+M356 lacked a particular bacterium that was present in the original microflora. Indeed, a *Clostridium thermosuccinogenes*-like bacterium, which was detected as a major band by denaturing gradient gel electrophoresis (DGGE) analysis of the original microflora (15), had not been isolated at the onset of the study.

In this report, we describe the construction of a cellulose-degrading, defined mixed culture (designated SF356) consisting of five bacterial strains which were dominant bacteria in the original microflora. This culture was found to stably coexist for more than 20 subcultures. We then constructed knockout communities in which one of the members was eliminated from SF356 in order to compare the SF356 knockout communities with the control in terms of both the stability for subcultures and the respective cellulose degradation processes. Furthermore, the characteristics of each of the members in the pure cultures were investigated in detail. The roles and relationships among the members of the community in situ were evaluated based on these results.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *C. straminisolvans* CSK1 (IAM 15070T, or DSM 16021T) was anaerobically isolated from the original microflora (21). *Clostridium* sp. strain FG4 was isolated from the original microflora in this study by using PYF-agar medium under anaerobic conditions. The PYF-agar medium contained the following components dissolved in 1 liter of water (pH 7.0): 10 g yeast extract, 10 g peptone, 5 g fructose, 400 mg NaHCO₃, 80 mg NaCl, 40 mg K₂HPO₄, 40 mg KH₂PO₄, 8 mg MgSO₄, 8 mg CaCl₂, and 15 g agar powder (Wako, Osaka, Japan). Stringent anaerobic procedures (17) were followed for the cultivation of *C. straminisolvans* CSK1 and *Clostridium* sp. strain FG4. Unless otherwise stated, *C. straminisolvans* CSK1 and *Clostridium* sp. strain FG4 were cultivated in medium 122 (20, 27) containing 0.5% (wt/vol) Avicel (Merck, Darmstadt, Germany) or cellobiose as a carbohydrate, respectively, under a 100% N₂ atmosphere without shaking at 50°C. *Pseudoxanthomonas* sp. strain M1-3, *Brevibacillus* sp. strain M1-5, and *Bordetella* sp. strain M1-6 were aerobi-

cally isolated from the original microflora (20). Unless otherwise noted, these aerobic strains were cultivated in PCS basal medium (pH 7.2) at 50°C under aerobic conditions. The PCS basal medium is the same as PCS medium (15) except that dried straw and CaCO₃ are omitted; i.e., the components dissolved in 1 liter of water are 1 g yeast extract, 5 g peptone, and 5 g NaCl.

Phylogenetic analysis. The 16S rRNA gene sequence of *Clostridium* sp. strain FG4 was determined by direct sequencing of the purified PCR-amplified 16S rRNA gene fragment as described previously by Kato et al. (20). Genomic DNA was extracted by the benzyl chloride method (34) and was used as the PCR template. PCR was performed with universal bacterial primers complementary to conserved regions of the 5' and 3' ends of the 16S rRNA gene, 27F (forward) (5'-AGAGTTTGATCCTGGCTCAG-3' [positions 8 to 27 according to *Escherichia coli* numbering]) and 1512R (reverse) (5'-ACGGCTACCTTGTACGACT-3' [positions 1512 to 1493 according to *E. coli* numbering]) (7). PCR was performed using AmpliTaq Gold (Applied Biosystems). The PCR products were purified with a QIAquick PCR purification kit (QIAGEN) according to the manufacturer's instruction. The purified 16S rRNA gene was sequenced directly using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) and an ABI PRISM model 377 genetic analyzer (Applied Biosystems). The obtained 16S rRNA gene sequences of isolated bacteria were compared with those from the DDBJ nucleotide sequence database using the program BLAST.

Mixed-culture systems. PCS-FP medium (pH 8.0) (PCS basal medium with 1% [wt/vol] filter paper and 2 g/liter of CaCO₃) in test tubes (16 by 100 mm for the 4.0-ml scale or 40 by 130 mm for the 70.0-ml scale) was used for the mixed-culture experiments. Each isolate was precultivated to the stationary phase under the conditions mentioned above. Forty microliters of the each preculture solution was inoculated into 4 ml of PCS-FP medium in various combinations (1st generations). These cultures were incubated under static conditions with a loose cap at 50°C. After 6 days of cultivation, the culture solution was mixed well, and then 40 or 700 μ l of culture solution was transferred to 4.0 or 70.0 ml of the same medium (2nd generation). The subcultures were then repeated in a manner similar to that of the 22nd generation.

Quantitative real-time PCR and specific PCR analyses. The genomic DNAs were extracted by the benzyl chloride method (34). The final purification was performed by RNase A treatment followed by polyethylene glycol treatment (28). The concentration of DNA was determined spectrophotometrically. Quantitative real-time PCR was carried out using a LightCycler system (Roche Diagnostics, Tokyo) with LightCycler-FastStart DNA Master SYBR green I (Roche Diagnostics) according to the methods described previously by Kato et al. (20). Specific primers for *C. straminisolvans* CSK1, *Pseudoxanthomonas* sp. strain M1-3, *Brevibacillus* sp. strain M1-5, and *Bordetella* sp. strain M1-6 were designed in our earlier work (20). A specific primer set for *Clostridium* sp. strain FG4 was newly designed using the program PRIMROSE (1) according to the sequence database of the Ribosomal Database Project II. The primer set was FG199f (5'-ATCACGGGGAGGCATCTTCC-3' [positions 183 to 199 according to *E. coli* numbering]) and FG442r (5'-CGTCACCTTCCTTCGTCCTC-3' [positions 442 to 461 according to *E. coli* numbering]). The specificity of all the primer sets was validated by real-time PCR; the quantitative signals detected for the targeted strain were not affected by presence of genome DNAs of the other strains. Real-time PCR was started with an initial denaturation step at 95°C for 10 min. Subsequently, target DNA was amplified in 45 cycles. Each cycle consisted of a denaturation step for 5 s at 95°C, an annealing step for 6 s at 68°C (for *C. straminisolvans* CSK1, *Clostridium* sp. strain FG4, and *Brevibacillus* sp. strain M1-5) or 10 s at 61°C (for *Pseudoxanthomonas* sp. strain M1-3 and *Bordetella* sp. strain M1-6), and an extension step for 20 s at 72°C. Fluorescence was detected at the end of each extension reaction. The temperature transition rate was 20°C/s except for the annealing temperature to the extension temperature, for which the rate was 2°C/s. The specificity of the amplified PCR product was assessed by performing a melting-curve analysis, which consisted of a denaturation step at 95°C, an annealing step for 15 s at 70°C, and gradual denaturation at a temperature transition rate of 0.1°C/s until 95°C had been reached and with the continuous detection of fluorescence. Two separate trials were conducted for the analysis of each sample. DNA mass-based standard curves for each isolate were generated with genomic DNA from each strain (1 pg to 10 ng of genomic DNA). Using these standard curves, the DNA mass for each strain present in the mixed-culture samples was calculated. The abundance ratios of the isolates were represented as the percentages of DNA mass, in which the sum of the DNA mass of all isolates is 100%.

The existence of each isolate in the mixed-culture systems was simply examined by detecting the amplification in 30 cycles of PCR (specific PCR analysis). The same primer sets used for the quantitative real-time PCR analysis were used for this specific PCR analysis. The PCR was initiated with an initial denaturation

step at 95°C for 10 min. Subsequently, the target DNA was amplified in 30 cycles. Each cycle consisted of a denaturation step for 30 s at 95°C, an annealing step for 30 s at 65°C (for *C. straminisolvans* CSK1, *Clostridium* sp. strain FG4, and *Brevibacillus* sp. strain M1-5) or 30 s at 55°C (for *Pseudoxanthomonas* sp. strain M1-3 and *Bordetella* sp. strain M1-6), and an extension step for 2 min at 72°C. The final extension step was performed for 5 min at 72°C. The PCR products were checked with 1.5% agarose gel.

Analyses of the process of filter paper degradation. For the analyses of filter paper degradation and metabolites, test tubes filled with 70.0 ml of PCS-FP medium were used. Two identical cultures of each bacterial combination were subjected to all analyses. In addition, all analyses of each sample were carried out in duplicate. The amount of residual filter paper was gravimetrically determined using a method described previously by Tailliez et al. (30). Uninoculated medium was used as a control. The soluble saccharide concentration in the culture solution was determined colorimetrically by the anthrone method (32). The acetate or ethanol concentration in the culture solution was determined enzymatically with an F-kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions.

Characterization of noncellulolytic isolates. The characteristics of the noncellulolytic isolates were assessed by cultivating the isolates in a cell-free culture filtrate of *C. straminisolvans* CSK1. *C. straminisolvans* CSK1 was anaerobically cultivated in PCS-FP medium for 8 days. The culture solution was then centrifuged, and the supernatant was sterilized by filtration with a 0.2- μ m-pore-size filter. Forty microliters of each precultured, noncellulolytic isolate was inoculated into 4 ml of the cell-free culture filtrate. Aerobic isolates were cultivated at 50°C under aerobic static conditions for 2 days. *Clostridium* sp. strain FG4 was cultivated at 50°C under anaerobic conditions for 5 days. Uninoculated culture filtrate was also subjected to the analyses as a control. After cultivation, saccharides, acetate, and ethanol concentrations were determined by the methods described above.

PCS basal medium and PCS-ethanol medium (PCS basal medium with 500 mg/liter of ethanol) were used for a test of the utilization of ethanol by *Brevibacillus* sp. strain M1-5. The utilization of carbohydrates by *Clostridium* sp. strain FG4 was assessed using medium 502 (see reference 9 and the DSMZ List of Media [<http://www.dsmz.de/media/med502.htm>]) containing 0.5% (wt/vol) of each substrate at 50°C under anaerobic conditions. The utilization of a substrate was judged from the decrease in the pH of the culture solution after 7 days of cultivation. Substrate utilization by the aerobic isolates was also assessed with the BIOLOG system (BIOLOG Inc., Hayward, CA) according to the manufacturer's instructions. The gram-positive strain (*Brevibacillus* sp. strain M1-5) was analyzed using GP MicroPlate, and gram-negative strains (*Pseudoxanthomonas* sp. strain M1-3 and *Bordetella* sp. strain M1-6) were analyzed using GN MicroPlate. A facultative anaerobe, *Pseudoxanthomonas* sp. strain M1-3, was also analyzed using AN MicroPlate under anaerobic conditions. Two identical cultures were subjected to all analyses. All analyses were performed in duplicate for each sample.

Nucleotide sequence accession number. The 16S rRNA gene sequence of *Clostridium* sp. strain FG4 has been deposited in the GenBank/EMBL/DBJ database under accession number AB207248.

RESULTS

Construction of a stable defined mixed culture degrading cellulose (SF356). *Clostridium* sp. strain FG4 was anaerobically isolated from the original microflora. The 16S rRNA gene sequence of *Clostridium* sp. strain FG4 was determined (GenBank accession number AB207248). The closest relative of *Clostridium* sp. strain FG4 was *Clostridium thermosuccinogenes* (accession number Y18180) on the basis of the 16S rRNA gene sequence (99.8% similarity), and the 16S rRNA gene sequence of *Clostridium* sp. strain FG4 was identical to the sequence of the DGGE bands detected from the original community (accession number AB077750), whose corresponding bacterium had not been isolated.

A five-strain mixed culture (designated SF356) consisting of *Clostridium* sp. strain FG4 and the members of CSK+M356 (*C. straminisolvans* CSK1, *Pseudoxanthomonas* sp. strain M1-3, *Brevibacillus* sp. strain M1-5, and *Bordetella* sp. strain M1-6) degraded cellulose effectively, even under aerobic conditions

FIG. 1. Relative abundance of each bacterium in SF356 and the knockout communities at day 6 of the 2nd and 22nd generations. The data show the results obtained from two independent subcultured communities (designated A and B). The values are expressed as the means of two replications of real-time PCR experiments. The variation between the real-time PCR experiments for the same DNA sample was less than 20%.

(5.29 ± 0.20 g/liter for 8 days), and exhibited similar functional and structural stability for subculture as those of the original microflora. After 20 subcultures, the efficiency of cellulose degradation by SF356 had not changed (5.12 ± 0.12 g/liter for 8 days), and no remarkable changes were found in the community structure of SF356 by quantitative real-time PCR analysis (Fig. 1). Hence, the five-strain mixed culture, SF356, was conceived as a model cellulose-degrading community.

Construction of knockout communities and stability for subculture. In order to elucidate the relationships among the members of SF356, mixed-culture systems in which one of the members was not included, namely, knockout communities, were constructed. Here, the knockout communities are represented as ΔX , where X is the strain name of an eliminated member (e.g., in $\Delta CSK1$, *C. straminisolvans* CSK1 was eliminated; in other words, $\Delta CSK1$ is a four-strain mixed culture composed of *Clostridium* sp. strain FG4, *Pseudoxanthomonas*

FIG. 2. Comparisons of the filter paper degradation processes of SF356 and the knockout communities. (A) Filter paper degradation over a period of 4 days (open bars) or 8 days (gray bars); (B) pH value; (C) accumulation of oligosaccharides; (D) accumulation of acetate; (E) accumulation of ethanol. Accumulation of the metabolites is represented as an increase in the metabolites during an 8-day period of cultivation (milligrams) per amount of filter paper degraded over a period of 8 days (grams). Values are expressed as the means of two culture solution samples. The error bars indicate standard deviations. Asterisks represent a significant difference ($P < 0.05$) from SF356. N.A., not applicable, because filter paper degradation did not occur.

sp. strain M1-3, *Brevibacillus* sp. strain M1-5, and *Bordetella* sp. strain M1-6).

The relative abundance of each bacterium in SF356 and the knockout communities before and after 20 subcultures was determined by real-time PCR. Figure 1 shows the relative abundance of each bacterium at day 6 of the 2nd and 22nd generations. As observed in SF356, no members disappeared, and the cellulose-degrading capabilities were maintained throughout the 20 subcultures in the case of both ΔM1-3 and ΔM1-6. On the other hand, in the other knockout communities, some members were not detected after 20 subcultures. For these unstable mixed cultures, the orders of disappearance of the members were investigated by specific PCR analysis. ΔCSK1, in which no cellulose degradation took place, was unstable for subculture. Three members disappeared during the progression of subcultures in the following order: *Clostridium* sp. strain FG4, *Brevibacillus* sp. strain M1-5, and *Pseudoxanthomonas* sp. strain M1-3. Finally, after 20 subcultures, only *Bordetella* sp. strain M1-6 remained (Fig. 1). In ΔM1-5, as was also observed in ΔCSK1, the relative abundance of *Bordetella* sp. strain M1-6 was remarkably high in the 2nd generation.

Two members of the community then disappeared during the progression of the subcultures in the following order: *C. straminisolvans* CSK1 and then *Clostridium* sp. strain FG4. Cellulose degradation disappeared in parallel with the disappearance of *C. straminisolvans* CSK1 (at the 10th or the 12th generation). Although the capability of ΔFG4 to degrade cellulose was not extinguished, ΔFG4 was also unstable for subcultures in terms of the structure; *Brevibacillus* sp. strain M1-5 disappeared during the progression of 20 subcultures (Fig. 1).

Filter paper degradation by SF356 and knockout communities. In order to elucidate the role(s) played by each bacterium in SF356, the processes of filter paper degradation in the 2nd generation of SF356 and its knockout communities were compared. Figure 2A shows the amount of filter paper degraded by the mixed cultures after 4 and 8 days of cultivation. No filter paper degradation took place in the case of ΔCSK1. ΔFG4 exhibited the most effective degradation of filter paper among the mixed cultures tested. This result suggested that *Clostridium* sp. strain FG4 has a negative effect on the degradation of cellulose. ΔM1-5 degraded cellulose more effectively than did SF356. ΔM1-3 and ΔM1-6 were not remarkably different from

TABLE 1. Pure culture of noncellulolytic isolates in PCS basal medium and in the cell-free culture filtrate of *C. straminisolvans* CSK1

Strain	PCS basal medium OD ₆₀₀ (SD) ^d	Culture filtrate of <i>C. straminisolvans</i> CSK1			
		OD ₆₀₀ (SD)	Saccharides (mg/liter) (SD)	Acetate (mg/liter) (SD)	Ethanol (mg/liter) (SD)
Uninoculated (before incubation)	0.006 (0.001) ^a	0.005 (0.002)	506.5 (39.9)	254.1 (2.0)	200.2 (14.5)
Uninoculated (after incubation)	0.009 (0.002)	0.007 (0.002)	520.5 (1.9)	275.6 (1.0)	158.5 (6.9)
<i>Clostridium</i> sp. strain FG4 ^b	0.048 (0.010)	0.110 (0.006)	241.0 (10.2)	461.3 (6.6)	205.0 (4.9)
<i>Pseudoxanthomonas</i> sp. strain M1-3	0.676 (0.161)	0.917 (0.038)	454.3 (0.9)	ND ^c	180.1 (2.1)
<i>Brevibacillus</i> sp. strain M1-5	0.733 (0.133)	0.940 (0.014)	536.0 (9.9)	609.0 (8.0)	2.4 (0.7)
<i>Bordetella</i> sp. strain M1-6	0.420 (0.040)	0.511 (0.006)	548.9 (14.4)	206.8 (13.2)	141.2 (4.9)

^a The values in parentheses are the standard deviations of two independent experiments.

^b *Clostridium* sp. strain FG4 was incubated under anaerobic conditions for 5 days, while the others were incubated under aerobic static conditions for 2 days.

^c ND, not detected.

^d OD₆₀₀, optical density at 600 nm.

SF356 in terms of the amount of filter paper that was degraded.

Figure 2B shows the transitions in the pH values in the mixed-culture solutions. The pH transition patterns of SF356, ΔM1-3, ΔM1-5, and ΔM1-6 were similar; the pH value decreased to around 6 and did not return to neutral. On the other hand, the pH value in ΔFG4 dropped once below 6 until day 3 and then returned to an approximately neutral value. The pH value in ΔCSK1 continued to increase to 9.0 until day 8.

The saccharides, acetate, and ethanol concentrations in the culture solutions of SF356 and the knockout communities were then determined. In order to exclude the effects of differences in cellulose degradation efficiency among the mixed cultures, the accumulation of the metabolites was expressed as relative values in which increases in metabolites during 8 days of cultivation (milligrams) were divided by the amount of degraded filter paper obtained after a culture period of 8 days (grams) (Fig. 2C to E). In ΔCSK1, no filter paper degradation took place, and therefore, no saccharides, acetate, or ethanol production was observed. The accumulation of saccharides in ΔFG4 was the highest and the accumulation of acetate and ethanol were the lowest among the mixed cultures tested. These results indicate that *Clostridium* sp. strain FG4 produced acetate and ethanol by fermenting saccharides. In ΔM1-5, the accumulation of saccharides was also significantly higher than it was in the case of SF356, thus indicating that *Brevibacillus* sp. strain M1-5 consumed saccharides. The accumulation of acetate in ΔM1-3 was the highest among the mixed cultures tested, indicating that *Pseudoxanthomonas* sp. strain M1-3 consumed acetate.

Characterization of the noncellulolytic members in pure culture. Each noncellulolytic isolate was cultivated in a cell-free culture filtrate of *C. straminisolvans* CSK1 to measure the growth and the concentrations of saccharides, acetate, and ethanol (Table 1). As a negative control, uninoculated filtrate was incubated and analyzed by the same manner (uninoculated [after incubation] in Table 1). In the filtrate, all of the isolates grew better than they had in PCS basal medium (Table 1). *Clostridium* sp. strain FG4 utilized saccharides and produced a large amount of acetate and a small amount of ethanol (Table 1). The capacity of *Clostridium* sp. strain FG4 to utilize cellobiose and glucose was also confirmed by acid production in medium 502 containing either cellobiose or glucose. *Pseudoxanthomonas* sp. strain M1-3 reduced acetate to a nondetectable level in

the filtrate (Table 1), although no positive reaction indicative of acetate utilization in the BiOLOG system was observed under either aerobic or anaerobic conditions. In addition, although no glucose or cellobiose utilization was detected by BiOLOG analysis, the saccharide concentration slightly decreased in the cultivation of *Pseudoxanthomonas* sp. strain M1-3 in the filtrate (Table 1). No consumption of saccharides was observed in the filtrate culture of *Brevibacillus* sp. strain M1-5, which utilized glucose but not cellobiose in BiOLOG culture. *Brevibacillus* sp. strain M1-5 reduced the ethanol concentration and increased the acetate concentration in the filtrate (Table 1). Moreover, in PCS-ethanol medium, a decrease in the ethanol concentration (111.9 ± 47.1 mg/liter after 2 days of cultivation and 436.2 ± 20.1 mg/liter in an uninoculated blank) and an increase in the acetate concentration (424.9 ± 16.1 mg/liter after 2 days of cultivation and 18.4 ± 1.6 mg/liter in an uninoculated blank) were simultaneously observed. The production of acetate in PCS basal medium (154.8 ± 15.6 mg/liter after 2 days of cultivation and 26.1 ± 9.2 mg/liter in an uninoculated blank) was significantly lower than that in the PCS-ethanol medium. These results confirmed that *Brevibacillus* sp. strain M1-5 has the capacity to utilize ethanol and to produce acetate from ethanol. *Bordetella* sp. strain M1-6 did not remarkably change with respect to the amount of saccharides, acetate, or ethanol in the filtrate (Table 1), and M1-6 was able to utilize a few substrates (e.g., certain amino acids) in the BiOLOG culture.

DISCUSSION

Construction of a stable defined mixed culture (SF356). We successfully isolated five strains of bacteria corresponding to all of the major bands detected by DGGE analysis from the original microflora. We then successfully constructed a defined mixed culture (designated SF356) that was able to effectively degrade cellulose and that exhibited functional and structural stability for subculture (Fig. 1); these properties were observed by mixing five strains of isolates. In the studies of pollutant degradation, it has been a common strategy to proceed as follows: (i) repeat the enrichment cultures in a medium containing the pollutant as the sole carbon (and/or nitrogen or energy) source, (ii) isolate the members, and (iii) cocultivate the isolates in order to investigate their functions and the relationships. Some reports have focused on the stable coex-

FIG. 3. Network model of substrate flow and the putative roles of each member in SF356. The solid lines indicate the flow of the substrates. The thickness of each line represents the relative contribution of a particular pathway in SF356. The dotted lines indicate promoting or inhibiting factors with respect to the efficiency of cellulose degradation. The names of the substrates are presented in boxes. The designations of the bacterial strains are presented in ovals.

istence of two bacterial strains in mutualistic or commensalistic association (e.g., see references 10 and 31). In addition, some groups have reported the construction of a defined mixed culture of more than two isolates in order to reveal metabolic sharing and synergistic interactions (2, 5, 6). For example, Dejonghe et al. (6) isolated bacteria corresponding to all of the major bands detected by DGGE analysis from a stable microflora that degrades the herbicide linuron, and they constructed a defined mixed culture that degraded linuron in order to reveal the relevant synergistic interactions. However, they did not make note of the stable coexistence of the members of the community. To the best of our knowledge, there appears to have been no reports to date regarding the construction of a defined mixed culture composed of more than two microorganisms that was able to stably coexist for a long period of time. Hence, our defined mixed culture, SF356, is considered a novel accomplishment, as it consists of dominant bacteria in an original microflora, and all of the members of the culture were able to coexist for a long period of time in a stable manner.

The putative roles of the members of SF356. By constructing and analyzing “knockout communities” in which one of the members was eliminated from SF356, the role played by each eliminated member in situ could be evaluated. Furthermore, the deduced roles (and in particular the capacity for substrate utilization) of each of the members were reexamined in pure-culture experiments. The putative roles played by the members in SF356, based on the results (including previously reported results) (20, 21), are summarized in Fig. 3.

(i) *C. straminisolvans* CSK1. *C. straminisolvans* CSK1 is an anaerobic cellulolytic isolate. Since cellulose degradation did not occur in the case of Δ CSK1, it was confirmed that *C. straminisolvans* CSK1 is a crucial bacterium for cellulose degradation in the SF356 community (Fig. 3). However, as discussed in our previous report (20), cellulose degradation did not occur in the pure culture of *C. straminisolvans* CSK1 under

aerobic static conditions, and the degradation efficiency was significantly low even under anaerobic conditions. These observations suggested that the contributions of aerobic noncellulolytic bacteria (i.e., the consumption of oxygen, pH neutralization, and stimulation of growth) (20) (Fig. 3) are essential for effective cellulose degradation by the community. In addition, the growth of noncellulolytic isolates was promoted in the cell-free culture filtrate of *C. straminisolvans* CSK1 (Table 1). Thus, the results are suggestive of a synergistic relationship between *C. straminisolvans* CSK1 and the aerobic noncellulolytic bacteria.

(ii) *Clostridium* sp. strain FG4. *Clostridium* sp. strain FG4 is an anaerobic noncellulolytic isolate. It appears that *Clostridium* sp. strain FG4 consumes saccharides derived from cellulose degradation and produces a large amount of acetate and a small amount of ethanol (Fig. 3), as based on the knockout experiments (Fig. 2C to E). This prediction was also supported by the results of the pure-culture experiments (Table 1).

Water-soluble oligosaccharides, especially cellobiose, are known to repress cellulase expression (18, 33), to inhibit cellulase activities (19, 23), and to inhibit cellulose degradation (24) by cellulolytic clostridia. Hence, the consumption of saccharides by *Clostridium* sp. strain FG4 is assumed to have a positive effect on achieving effective cellulose degradation. However, although the accumulation of saccharides in Δ FG4 was higher than that in the case of SF356 (Fig. 2C), Δ FG4 exhibited more effective degradation of filter paper than SF356 (Fig. 2A). This observation indicated that *Clostridium* sp. strain FG4 has a negative effect on cellulose degradation, and this effect exceeded the positive effects exerted by the saccharide scavenge. Although the pH value in Δ FG4 dropped once below 6 before day 3, it returned to an approximately neutral value, and the acidic pH in SF356 (the mixed culture containing *Clostridium* sp. strain FG4) was generally maintained during the cultivation period (Fig. 2B). The production of acetic acid by *Clostridium* sp. strain FG4 would be expected to maintain an acidic pH. This could account for the negative effects on cellulose degradation exerted by *C. straminisolvans* CSK1, which exhibited only slight cellulose degradation under a pH value of 6.0 (21).

(iii) *Pseudoxanthomonas* sp. strain M1-3. *Pseudoxanthomonas* sp. strain M1-3 is a facultative anaerobe and is considered to greatly contribute to oxygen consumption, pH neutralization, and stimulation of growth of *C. straminisolvans* CSK1 (20) (Fig. 3). Since the accumulation of acetate in Δ M1-3 was the highest among the mixed cultures tested (Fig. 2D), it is expected that *Pseudoxanthomonas* sp. strain M1-3 consumes acetate in SF356. Acetate utilization was not detected in *Pseudoxanthomonas* sp. strain M1-3 by the BiOLOG system. However, a significant decrease in the acetate concentration was observed in the cell-free culture filtrate of *C. straminisolvans* CSK1, which would be expected to imitate the medium composition during cellulose degradation by mixed cultures in PCS-FP medium. The consumption of acetate by *Pseudoxanthomonas* sp. strain M1-3 would also be expected to alleviate the negative effects of acetate that lead to a drop in pH and inhibit cellulose degradation.

Pseudoxanthomonas sp. strain M1-3 exhibited only a slight consumption of saccharides in the cell-free culture filtrate of *C. straminisolvans* CSK1 (Table 1). This result is consistent

with the detection of exoglucanase and cellobiase activities in the culture supernatant and cell extract, respectively, of *Pseudoxanthomonas* sp. strain M1-3 (data not shown). However, since the accumulation of saccharides in Δ M1-3 was not high (Fig. 2C), the contribution in the context of SF356 was thought to be slight at most.

(iv) ***Brevibacillus* sp. strain M1-5.** *Brevibacillus* sp. strain M1-5 is an obligate aerobe and is considered to contribute to oxygen consumption and pH neutralization (20), as does *Pseudoxanthomonas* sp. strain M1-3. The putative roles of *Brevibacillus* sp. strain M1-5 are summarized in Fig. 3. Since the accumulation of saccharides in Δ M1-5 was significantly higher than that in SF356 (Fig. 2C), *Brevibacillus* sp. strain M1-5 was assumed to consume saccharides. Indeed, the capability to utilize glucose (but not cellobiose) was observed with the BiOLOG system. On the other hand, no consumption of saccharides was observed in the cell-free culture filtrate of *C. straminisolvens* CSK1 (Table 1). A likely explanation for the lack of saccharide consumption was that the saccharides present in the filtrate were in the form of oligosaccharides rather than glucose. Indeed, the glucose concentration in the original microflora was reported as being less than 10 mg/liter, whereas approximately 600 mg/liter of total soluble saccharides was detected (15).

A remarkable decrease in the ethanol concentration as well as a remarkable increase in the acetate concentration were observed in the cell-free culture filtrate of *C. straminisolvens* CSK1 (Table 1). Furthermore, the acetate production of *Brevibacillus* sp. strain M1-5 was enhanced in PCS-ethanol medium compared with that in PCS basal medium. These results suggested that *Brevibacillus* sp. strain M1-5 produces acetate from ethanol. However, the contribution of these products to the entire reaction in SF356 is not expected to be substantial, since there was little difference in the amount of either acetate or ethanol in SF356 and Δ M1-5 (Fig. 2D and E).

(v) ***Bordetella* sp. strain M1-6.** *Bordetella* sp. strain M1-6 is considered to contribute to oxygen consumption and pH neutralization (20) in a manner similar to that of *Pseudoxanthomonas* sp. strain M1-3 (Fig. 3). *Bordetella* sp. strain M1-6 is considered to be independent of the substrates derived from cellulose degradation, i.e., saccharides, acetate, and ethanol. *Bordetella* sp. strain M1-6 appeared to be completely dependent on the peptides or amino acids contained in the PCS basal medium.

Interspecies relationships among members in SF356. It was important to examine here the reason for which the five strains were able to coexist in a stable manner; along these lines, elucidation of the interspecies relationships among the members was expected to provide insight into the observed stability of this community. In the present study, the impact of the elimination of one member on the other members of the community was evaluated by analysis of the dynamics of the members in knockout communities as they progressed through 20 subcultures (Fig. 1). In Δ CSK1, in which cellulose degradation did not occur, three members disappeared through the subcultures in the following order: *Clostridium* sp. strain FG4, *Brevibacillus* sp. strain M1-5, and *Pseudoxanthomonas* sp. strain M1-3. This finding gave the following estimates that these three strains, especially *Clostridium* sp. strain FG4, strongly depend on *C. straminisolvens* CSK1 and presumably on metab-

olites from the degradation of cellulose. This assumption is consistent with the putative roles of the members discussed above (Fig. 3); these three members were found to utilize substrates derived from cellulose degradation, namely, saccharides and acetate. Likewise, *Brevibacillus* sp. strain M1-5 disappeared from Δ FG4, indicating that *Brevibacillus* sp. strain M1-5 depends on *Clostridium* sp. strain FG4. Indeed, *Brevibacillus* sp. strain M1-5 can utilize glucose but not cellobiose. *Clostridium* sp. strain FG4 would be expected to supply glucose for *Brevibacillus* sp. strain M1-5 by the extracellular degradation of oligosaccharides.

C. straminisolvens CSK1 disappeared through the subcultures in Δ M1-5. This unexpected result cannot be explained by the putative roles of the members of the community discussed above. One possible key to this instability could be that the relative abundance of *Bordetella* sp. strain M1-6 was remarkably high in the 2nd generation (Fig. 1). It is predicted that *Brevibacillus* sp. strain M1-5 has suppressive effects on the growth of *Bordetella* sp. strain M1-6. This type of suppressive (or competitive) relationship is assumed to be essential for the stability of SF356.

Several reports have described various types of microbial interspecies relationships, such as antagonism, competition, commensalism, and symbiosis between two microorganisms. However, there appears to have been no report to date to demonstrate that these relationships actually operate in complex communities and how they affect the structure, function, and stability of complex communities. The evaluation of the total network of interspecies relationships would be important to understand such complex communities. In this study, we successfully constructed a stable defined mixed culture (SF356). In this stable community, we were able to identify various intertwined relationships not only by the detection of synergistic or commensalistic interactions but also by detecting inhibitory effects (such as the inhibition of cellulose degradation by the production of an excess amount of acetic acid) and competition (such as that between *Brevibacillus* sp. strain M1-5 and *Bordetella* sp. strain M1-6). It appears that achieving a balance of various types of relationships (both positive and negative) is essential for the stable coexistence of the members in SF356.

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