ISSN 0026-2617, Microbiology, 2009, Vol. 78, No. 2, pp. 234–239. © Pleiades Publishing, Ltd., 2009.
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EXPERIMENTAL ARTICLES

A Cellulose-Decomposing Bacterial Association

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Received January 28, 2008

Abstract—The structure of a cellulose-decomposing bacterial association was described using phenotypic and phylogenetic characteristics. Based on their morphological, physiological, and biochemical characteristics, the bacteria isolated from the association were identified as *Sporocytophaga* sp., *Xanthomonas* sp, and *Pseudomonas* sp. The phylogenetic analysis based on comparison of 16S rRNA gene fragments obtained from the association revealed six bacterial species belonging to the clusters of *Alcaligenes* sp., *Ochrobactrum* sp., *Sphingomonas* sp., *Achromobacter* sp., *Pseudomonas* sp., and *Flexibacteriaceae* (*Sporocytophaga*).

Key words: bacterial associations, cellulose, *Sporocytophaga* sp.

DOI: 10.1134/S0026261709020155

Cellulose constitutes on the average 30 to 50% of plant biomass and is the main carbon source delivered to soil with plant debris [1, 2].

Under natural conditions, cellulose is decomposed only by bacteria, actinomycetes, and fungi producing specific hydrolytic enzymes, cellulases. This process is an important part of the biosphere's carbon cycle.

The genes encoding cellulases are subject to catabolite repression; the synthesis of these enzymes is inhibited by accumulation of glucose, the end product of cellulose hydrolysis [2]. Cellulose decomposition therefore stops.

In soil and other natural environments, cellulose-decomposing bacteria exist together with other microorganisms that utilize glucose and cellobiose, the products of cellulose hydrolysis, as carbon and energy sources [3–5]. Catabolic depression of the cellulase genes is therefore relieved and cellulose decomposition continues due to the de novo synthesized enzymes and increased biomass of cellulolytic bacteria. Cellulose-decomposing microorganisms are believed to play the key role in the formation of the soil microbial complex [6]. The difficulty of separating cellulose-decomposing bacteria from accompanying microflora is well known; a certain symbiosis between these organisms has been suggested at the early stages of investigation [3, 7]. However, few publications deal with this issue. Cellulose decomposition by pure cultures of *Cellulomonas flavigena* was shown to be less efficient than in mixed cultures [4]. Some authors reported an exchange of growth factors between cellulose-decomposing bacteria and accompanying microorganisms, which enabled their growth and higher rates of cellulose degradation [3]. Growth of *Azotobacter* sp. and nitrogen fixation were observed in a cellulose-utilizing mixed bacterial culture [7]. Growth and nitrogen fixation were reported also in mixed cultures of azospirilla and cellulose-decomposing bacteria [8].

These results suggest complex, multifunctional interactions in associations of cellulolytic bacteria and other microorganisms.

Among the organisms isolated from aerobic cellulolytic enrichments, gram-negative bacteria of the genera *Xanthomonas*, *Pseudomonas, Alcaligenes*, and *Aerobacter* were revealed, as well as gram-positive *Micrococcus* [4, 9, 10]. However, the published data are insufficient for the understanding of stability of such associations and of the specificity of their composition related to certain species of cellulose-decomposing bacteria.

The goal of the present work was to investigate the structure of a cellulose-utilizing microbial association formed in a *Sporocytophaga* sp. enrichment culture.

MATERIALS AND METHODS

Isolation and maintenance of a cellulose-utilizing microbial association. The cellulose-utilizing microbial association was isolated from the peat–straw substrate obtained by composting peat with straw. The isolation was carried out by transfers of the dilutions of the suspended peat–straw substrate on agarized Hutchinson medium with a cellulose filter (Ekros, blue band). The medium contained the following (g/l): $K_2HPO_4 \cdot 3H_2O$, 1.0; $CaCl_2 \cdot 2H_2O$, 0.01; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.1; $FeCl_3 \cdot 6H_2O$, 0.01; $NaNO_3$, 2.5; agar, 20.0 (pH 7.2–7.3). The plates were incubated at 26°C. After seven days of incubation, cellulose-utilizing microbial

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associations were revealed as lighter zones of cellulose decomposition. Cellulose-decomposing bacteria from the zone with the most pronounced cellulose hydrolysis and dark yellow pigmentation were transferred to fresh agarized medium of the same composition and incubated under the same conditions.

The association thus obtained completely decomposed the paper filter in a month; it was maintained for a year by monthly transfers to fresh Hutchinson medium with filter paper.

The stability of the association was assayed as the preservation of the qualitative and quantitative composition of heterotrophic bacteria in a year after two sequential transfers. For this purpose, bacteria were transferred to glucose–peptone agar (GPA) containing the following (g/l): glucose, 1.0; peptone (Serva), 1.0; yeast extract, 1.0; $K_2HPO_4 \cdot 3H_2O$, 1.0; agar, 20.0. The plates were incubated at 26°C. Diversity of heterotrophic bacteria was determined after five days of incubation.

Together with the description and enumeration of the heterotrophic morphological types, isolation of the members of this association was carried out using generally accepted techniques.

In order to determine the bacteria capable of growth on cellulose, all the isolates were inoculated on agarized Hutchinson medium with filter paper and incubated for a month at 26°C.

Only one of the isolates obtained from the association was able to decompose cellulose. Microscopy and control transfers revealed that it was a mixed culture containing a cellulose-decomposing bacterium of the genus *Sporocytophaga*. Attempts to isolate the individual strains were unsuccessful. Since *Sporocytophaga* sp. is resistant to high temperatures, pasteurization and subsequent cloning on Hutchinson mineral medium with filter paper were used to obtain it in pure culture. The purity of the culture was confirmed by plating on glucose–peptone agar and Hutchinson medium, as well as by direct microscopy.

After the isolation of pure cultures, bacteria were identified on the basis of their morphological, cultural, physiological, and biochemical characteristics.

Determination of the composition of the cellulolytic association by 16S r RNA gene sequences. Total DNA was isolated from the association in two sequential transfers [11]. The results for each transfer were analyzed separately. The culture of the cellulose-degrading bacterial association was lysed in a buffer (0.2 M NaOH, 1%, SDS). The lysate was heated for 5 min at 95°C, and the polymerase chain reaction (PCR) was carried out. For PCR, the following primers were used: forward primer fBD1 (5'-HAATHYGTGCCAGCAGC-3') and reverse primer rBD1 (5'-GTCRTCCYDCCTCCTC-3'); the primers were developed in 2005 [12].

The PCR products were cloned in the pTZ57R vector (Fermentas). For this purpose, ligation of the amplified DNA fragments and transformation of *E. coli* DH10B cells with the obtained constructs were carried out. The transformants were screened by white–blue selection. White colonies, expected to contain inserts of the PCR product, were analyzed by PCR with the M13 primers (Fermentas) to the phage M13 DNA sequences flanking the insert.

In order to identify individual clones, restriction analysis was carried out. For this purpose, 5 μl of the PCR fragments were added to 5 μl of the reaction mixture containing the reaction buffer (Fermentas) and *Hae*III restriction endonuclease (Sigma), 2 U/ml; the mixture was then incubated at 37°C for 2 h. The results of restriction were visualized by electrophoresis in 4% agarose gel.

After determination of the clones containing different 16S rRNA genes, the isolated genes were sequenced. For this purpose, plasmid DNA was isolated and purified as follows: a 24-h culture of transformed *E. coli* cells was grown at 37°C, precipitated in 1.5-ml centrifuge tubes at 18 000 *g* for 1–2 min, and washed with water. The pellet was resuspended in 200 μl of buffer solution 1 (50 mM Tris–HCl, pH 7.8; 10 mM EDTA, and 100 μg/ml RNase A (Fluka)) under shaking.

To the resulting homogeneous suspension, 200 μl of solution 2 was added (0.2 M NaOH, 1% SDS); the mixture was gently mixed and incubated for 3–5 min at room temperature to clarification. The chromosomal DNA was precipitated with solution 3 (1.32 M CH_3COOK, pH 4.8); after gentle mixing and incubation for 5 min at –20°C, the mixture was centrifuged for 15 min at 18000 *g* (4°C).

The plasmid DNA was purified by hydrophobic chromatography. Syringes (2 ml) were inserted into the mini-columns and connected to the vacuum pump. The supernatant was transferred into the syringes; 1 ml of the resin (Promega) was added, and plasmid DNA with the resin was precipitated under vacuum. The columns were washed with 2 ml of solution 4 (80 mM CH_3COOK; 8.3 mM Tris–HCl, pH 7.5; 40 μM EDTA; 55% ethanol). They were then disconnected from the vacuum system and transferred to 1.5-ml test tubes. The washing solution was removed by centrifugation for 5 min at 18000 *g*. The plasmid DNA was then washed off the columns by adding 50 μl of water (70°C) and centrifugation of the columns together with the tubes for 20 s at 15000 *g*.

The sequencing was carried out in a CEQ8000 automatic sequencer (Beckman Cultier); the Beckman reaction kit was used according to the manufacturer's recommendations. The Vector NTI 8 software package was used to analyze the results of sequencing. The microorganisms were identified by comparison of the nucleotide sequences of the PCR fragments of 16S rRNA genes with the sequences stored in the NCBI database using the nBLAST protocol.

Table 1. Characterization of bacteria within the association

Morphotype	Gram reaction	Hugh and Leifson's test	Oxidase	Catalase	Cellulose hydrolysis	Bacterial names
01	–	O*	+	+	+	*Sporocytophaga* sp. + Unknown species
02	–	O	–	+	–	*Xanthomonas* sp.
03	–	Not determined	+	+	–	Lost
04	–	O	+	+	–	*Pseudomonas* sp.

* O indicates aerobic type of metabolism.

Table 2. Content and frequency of occurrence for the morphotypes of heterotrophic bacteria within the cellulose-decomposing association

Morphotype	Transfer 11		Transfer 12	
	Content, CFU/cm^2	Frequency of occurrence, %	Content, CFU/cm^2	Frequency of occurrence, %
01	$(0.8 \pm 0.08) \times 10^8$	0.3	$(1.1 \pm 0.2) \times 10^8$	0.2
02	$(6.6 \pm 0.2) \times 10^8$	1.3	$(8.0 \pm 0.8) \times 10^8$	1.4
03	$(3.0 \pm 0.2) \times 10^{10}$	57.4	$(3.3 \pm 0.2) \times 10^{10}$	58.5
04	$(2.1 \pm 0.1) \times 10^{10}$	41.0	$(2.2 \pm 0.1) \times 10^{10}$	39.9

$F_{0.95} = 1.54$; $p\% = 4.5$.

RESULTS AND DISCUSSION

The cellulose-decomposing microbial association isolated from the peat–straw compost was maintained for a year on agarized Hutchinson medium with filter paper. Growth on filter paper occurred as fuzzy zones of dark yellow color. The maceration of the filter was visible after a week of incubation; in a month, cellulose was almost completely decomposed. Transfer of the material from decomposing paper on glucose–peptone agar resulted in growth of four types of bacteria. No fungi or actinomycetes were revealed. Bacteria colonies obtained on the agar were isolated as individual cultures, checked for purity, and maintained on GPA. All the isolates were strictly aerobic, asporogenic gram-negative bacteria.

The isolates of the morphological type 01 were a mixed culture. Attempts to separate its components by repeated cloning on solid media were unsuccessful. Microscopy of the colonies and of fuchsin-stained slides revealed the presence of bacteria morphologically resembling *Sporocytophaga* sp.

These bacteria were isolated in pure culture by pasteurization. On mineral medium with filter paper, the isolate exhibited characteristic growth as fuzzy colonies surrounded by the zones of cellulose hydrolysis. Microscopically, the isolate consisted of single cells (up to 10 μm in length) with rounded ends. The Gram reaction was negative. In old cultures, numerous cysts of characteristic *Sporocytophaga* morphology were found. On media with 0.1% glucose, the culture grew as big mucous convex colonies; microscopy revealed bacteria morphologically similar to *Sporocytophaga* sp.

Isolates of the morphotype 03 grew very poorly on agarized media and were lost after two transfers.

Other isolates were aerobic asporogenic gram-negative bacteria. On the basis of their morphological, cultural, physiological, and biochemical characteristics, the morphotype 02 isolates were classified as *Xanthomonas* sp., and the morphotype 04 isolates, as *Pseudomonas* sp. [13]. None of them were able to decompose cellulose (Table 1).

Thus, the cellulose-decomposing bacterial association contained at least four bacterial species; only three of them were isolated and identified according to their phenotypic characteristics. *Sporocytophaga* sp. was the key organism forming this association.

In order to assay the stability of this association, its qualitative and quantitative composition was determined a year after isolation. Identical bacterial species, including those not maintained in a pure culture (morphotype 03) were obtained after 11 to 12 transfers of the association. The results are presented in Table 2.

Statistical analysis of the data did not reveal any difference between these samples ($F_{0.95} = 1.54$). Thus, the composition of the culture remained stable after 12 transfers. This finding was confirmed by molecular genetic analysis of 16S rRNA genes of the association.

The library of 16S rRNA genes from the cellulose-decomposing bacterial association was formed of 87 *E. coli* clones transformed by the pTZ57R vector containing the relevant genes.

Restriction analysis revealed that the cellulose-decomposing association contained six different 16S rRNA sequences. The restriction profiles for the trans-

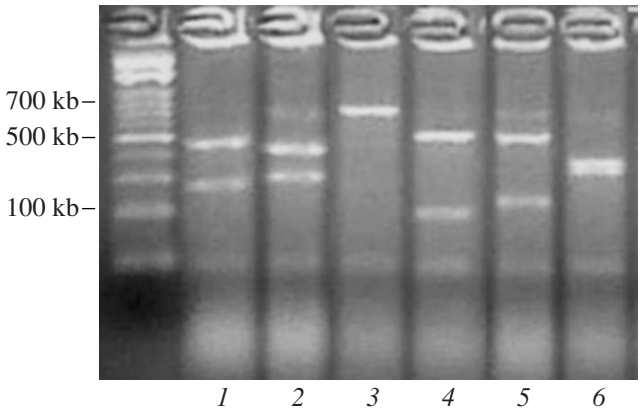
fers 11 and 12 were similar, indicating stability of the association. This result confirms the data obtained by traditional techniques (figure).

Each of the 16S rRNA genes was sequenced for comparison with the NCBI international database of bacterial 16S rRNA sequences. The results are presented in Table 3.

The cellulose-decomposing association contained at least five bacterial species similar to the members of the genera *Sphingomonas, Ochrobactrum, Alcaligenes, Pseudomonas*, and *Achromobacter*, as well as a member of the family *Flexibacteriaceae*, phylum BXX *Bacteroidetes.* No sequence present in the database exhibited high similarity to the latter sequence required for genus identification, i.e., above 95%.

As was stated above, only one species capable of cellulose hydrolysis was isolated from this association; its phenotypic characteristics supported its classification as *Sporocytophaga* sp.

According to the phylogenetic classification of bacteria, the genus *Sporocytophaga* belongs to the phylum BXX *Bacteroidetes* of the family *Flexibacteriaceae.* The similarity between the new sequence and the known 16S rRNA gene sequences of this genus is low. However, the NCBI database contains only six 16S rRNA gene sequences for *Sporocytophaga*; the similarity between some of them does not exceed 80%, which is also insufficient for genus identification (these organisms were initially identified using morpho-cultural and physiologo-biochemical characteristics). This bacterial group is therefore insufficiently studied and requires further investigation. The "paradoxical" nature of the phylum BXX *Bacteroidetes* [14] manifested in different levels of divergence for bacterial phenotypes and genotypes, as well as poor knowledge of the genetics of this bacterial genus support our classification of the cellulose-decomposing bacterium isolated from the association as *Sporocytophaga* sp. based on its phenotypic characteristics.



Restriction profiles of 16S rRNA genes (*1–6*) from the bacteria of the cellulose-decomposing association (*Hae*III restriction endonuclease).

Other bacteria identified by their 16S rRNA gene sequences are the satellites of *Sporocytophaga* sp.; they utilize the products of cellulose hydrolysis as the main source of carbon and energy.

Comparative results on the biodiversity of the cellulose-decomposing bacterial association obtained by isolation of the microorganisms on nutrient media with subsequent analysis of their phenotypic characteristics, as well as by molecular genetic analysis of 16S rRNA gene sequences are presented in Table 4.

Three out of six bacterial species detected by the 16S rRNA gene analysis were obtained in pure culture. Only two of these (*Pseudomonas* sp. and *Sporocytophaga* sp.) corresponded to the species identified by their phylogenetic position. The identity of *Xanthomonas* sp. is questionable. It should be noted that the authors who have analyzed the nucleotide sequences of 16S rRNA

Table 3. Composition of the cellulose-decomposing bacterial association identified by 16S rRNA gene sequences

Profile no.	NCBI accession no.	Closest relatives in NCBI database	Similarity, %*
1	EU697496	*Sphingomonas* sp. JRL-5 AF181572	99
		Sphingomonas sp. strain: MBIC3365 AB015049	99
2	EU697494	*Ochrobactrum* sp. BMC4 AB272338	97
		Uncultured *Ochrobactrum* sp. clone 60-3-17 DQ842539	97
3	EU684264	*Alcaligenes* sp. Q-6 AB246810	98
		Alcaligenes sp. mp-2 AY331576	98
4	EU697495	*Sporocytophaga* sp. A61 AM179866	82
		Family *Flexibacteriaceae*	100
5	EU697497	*Pseudomonas* sp. 5A AF411854	99
		Pseudomonas sp. BSi20329 DQ492750	99
6	EU697493	*Achromobacter* sp. LQX-8 DQ211691	98
		Uncultured *Alcaligenes* sp. AJ306836	98

Note: * Similarity (%) between 16S rRNA gene sequences from the association and the closest gene from the NCBI database.

Table 4. Bacterial biodiversity within the cellulose-decomposing association

Bacterial association identified by phenotypic characteristics		Bacterial association identified by 16S rRNA gene sequences	
Isolate	Bacteria	16S rRNA profile	Bacteria
01	*Sporocytophaga* sp. + uncultured	4	Family *Flexibacteriaceae* (*Sporocytophaga* sp.)
02	*Xanthomonas* sp.	1	*Sphingomonas* sp.
03	Not identified (weak growth on the nutrient medium)		
04	*Pseudomonas* sp.	5	*Pseudomonas* sp.
		2	*Ohrobactrum* sp.
		3	*Alcaligenes* sp.
		6	*Achromobacter* sp.

genes from various *Xanthomonas* sp. strains classified them within the genus *Sphingomonas* [15]. Moreover, the phenotypic characteristics of the *Xanthomonas* sp. strain isolated from the association coincide completely with the characteristics of the genus *Sphingomonas* as described by Takeuchi et al. [16]; this genus is not included in the 1997 edition of the Bergey's Manual of Systematic Bacteriology.

The distribution of *Sphingomonas* sp. in the environment is poorly studied. There is evidence of their presence in the activated sludge of wastewater treatment facilities and in municipal wastewater [17], as well as in soils [18]. Bacteria of this genus exhibit high metabolic activity; they decompose lignin and stable organic xenobiotics [18, 19]. Their presence in the cellulose-decomposing bacterial association is therefore of interest considering efficient functioning of this association under environmental conditions where cellulose is present mostly as a stable lignocellulose complex. Their ability to decompose stable organic xenobiotics in mixed culture with cellulose-decomposing bacteria may be important for soil self-purification from xenobiotics.

Bacteria of the genus *Ochrobactrum* are even less studied. In the 1997 Bergey's manual, only one species *Ochrobactrum anthropi* was described, initially isolated from clinical material. However, bacteria of this genus were recently revealed in soil and wheat rhizoplane, as well as in lupine root nodules [20] and rice root tissues [21]. Their presence in the cellulose-decomposing bacterial association described in the present work is difficult to explain. These organisms, located in the rhizoplane or plant tissues, probably arrive to the soil with plant debris and are incorporated into the formed bacterial association in the course of cellulose decomposition. Functionally, they are most probably satellites of *Sporocytophaga* sp., utilizing the metabolic products of these organisms as sources of energy, nutrients, and growth factors.

Thus, prolonged cultivation of a microbial enrichment culture on the medium with cellulose as the only carbon source resulted in formation of a stable microbial association. *Sporocytophaga* sp. was shown to be the key element of this association; only these organisms are capable of cellulose hydrolysis. Other bacteria in the association did not decompose cellulose and did not use it as a source of carbon and energy. Their survival was therefore completely dependent on the efficiency of cellulose hydrolysis and of the amount of resulting hydrolysis products (glucose and cellobiose); under experimental conditions, the latter were the only source of energy and carbon. The question of the mode of interaction between *Sporocytophaga* sp. satellites is therefore natural. Analysis of the CFU data for the association demonstrates a distinct distribution of different microbial forms according to the frequency of their occurrence in the association (Table 2). Most of the bacteria belong to the genus *Pseudomonas* and to an unidentified bacterium growing poorly on this medium. Thus, the qualitative and quantitative composition of bacteria within the association is highly structured and based on trophic and chorological relations of a complex character. The relations between the microorganisms within this association are probably harmonized by mutual exchange of growth factors and nutrient components; thus, they may be considered a form of associative symbiosis. Stability of the association's composition (at least under experimental conditions) indicates the absence of competition between these organisms.

ACKNOWLEDGMENTS

The authors are grateful to E.E. Andronov and A.G. Pinaev, All-Russia Research Institute of Agricultural Microbiology, Russian Academy of Agriculture, for consultations and assistance in molecular genetic analysis.

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