Biosci. Biotech. Biochem., **58** (3), 600–601, 1994

Isolation of *Sphingomonas* Strains from Ears of Rice and Other Plants of Family Gramineae

Kazuyoshi KAWAHARA, Ikuko MIZUTA, Wakana KATABAMI, Megumi KOIZUMI, and Sachio WAKAYAMA*

Department of Bacteriology, The Kitasato Institute, 5–9–1, Shirokane, Minato-ku, Tokyo 108, Japan
**Kibun Foods Inc., 7–14–13, Ginza, Chuo-ku, Tokyo 104, Japan*
Received October 25, 1993

***Sphingomonas* strains were isolated in high frequency from ears of rice (*Oryza sativa*), *Echinochloa crus-galli*, and *Setaria viridis*. Isolates were identified by the rapid method of cellular fatty acid analysis. Isolated *Sphingomonas* strains have 2-hydroxymyristate as a sole hydroxy fatty acid, ubiquinone Q-10, and glycosphingolipid. This study demonstrated that sphingomonads are members of a natural flora of microorganisms in ears of rice and taxonomically related plants.**

The genus *Sphingomonas* was first proposed by Yabuuchi *et al.*[1] and composed of 5 species including *S. paucimobilis* as the type species. Most of the strains used in their study were isolated from patients or from clinical materials. However, *S. paucimobilis*, formerly classified as *Pseudomonas paucimobilis*, were known to be isolated from non-clinical environments[2] and also from rhizospheres of rice plants.[3–5] We have demonstrated that *S. paucimobilis* does not contain lipopolysaccharide,[6] which is the major component of the outer membrane of Gram-negative bacteria, and glycosphingolipid plays a similar role in the outer membrane of this bacterium.[7] Those exciting findings have led us to the idea that its unusual cell surface might have some relationship to the environment where the bacterium is living. The early study done by the group of Komagata had already showed that *S. paucimobilis* and similar bacteria were often isolated not only from rhizospheres but also from ears of rice (H. Oyaizu and K. Komagata, personal communication). Based on this knowledge we have tried to isolate sphingomonads from the ears of rice and other plants of the family Gramineae to find out the native living environment of this peculiar bacterium.

Plant samples were collected from Tokyo, Kanagawa, Saitama, Ibaraki, and Chiba prefectures from May to October in 1992. Samples were washed once with salt solution containing 0.2% $(NH_4)_2SO_4$, 0.2% K_2HPO_4, and 0.01% $MgSO_4 \cdot 7H_2O$, and ground with the same solution (200 mg sample/2 ml). The sample suspensions were diluted to make a 10-fold dilution series, and 0.1 ml of the diluted suspension was spread onto agar plates. The agar medium contained 0.5% glucose, 0.5% Yeast Extract (Difco), 0.5% Casamino acids (Difco), and 12.5 μg/ml polymyxin B (Wako Pure Chemicals). Polymyxin B was added to the medium because type strains of *S. paucimobilis* and *S. capsulata* were resistant to this drug (MIC; 50 μg/ml), and this drug was known to be effective to the lipopolysaccharide-containing Gram-negative bacteria. The agar plates were incubated for 2 days at 30°C, and the bacterial colonies were picked up. After purification the isolates were inoculated in 5 ml of the medium described in our previous paper[8] in screw-capped glass tubes, and incubated at 30°C with shaking for 24 h. After incubation, cells were harvested by centrifugation, and hydrolyzed directly with 2 ml 4 M HCl at 100°C for 5 h in the same tube used for cultivation. Free fatty acids were extracted, concentrated by evaporation, methyl-esterified by diazomethane, and analyzed by gas-liquid chromatography (GLC) (Shimadzu GC-14A) with a 25-m capillary column, Shimadzu CBP-1. All procedures for the fatty acid analysis could be completed within one day.

From ears of wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) collected in May, 82 colonies were isolated. Among them no bacterium showed the *Sphingomonas*-type profile of cellular fatty acids. In September and October ears of rice (*Oryza sativa*), *Echinochloa crus-galli*, and *Setaria viridis* were collected and used for the experiments. When agar plates containing polymyxin B were used for isolation, 46 strains out of 124 isolates showed the characteristic profile of fatty acids of *Sphingomonas*. In another experiment, isolation was done on agar plates without polymyxin B. In this case 8 strains from 43 isolates were found to have the

Table Chemotaxonomical Characteristics of the *Sphingomonas*-like Isolates from Ear of Plants

Strains	Source of isolation	Color of colony	Cellular fatty acids (%)					Ubiquinone	Glycosphingolipid	
			C14:0	C16:1	C16:0	C18:1	2-OHC14:0		Monosacch.[a]	Oligosacch.[b]
MK251	*O. sativa*	Yellow	9	1	20	57	13	Q-10	+	+
MK255	*O. sativa*	Yellow	12	2	18	58	10	Q-10	+	+
MK329	*E. crus-galli*	Yellow	2	9	18	58	10	Q-10	+	−
MK333	*O. sativa*	Yellow	11	1	26	51	11	Q-10	+	+
MK341	*S. viridis*	Pink	Trace	10	16	53	21	Q-10	+	+
MK343	*S. viridis*	Yellow	4	2	15	57	22	Q-10	+	+
MK346	*E. crus-galli*	Yellow	Trace	2	17	57	24	Q-10	+	+
MK347	*E. crus-galli*	Yellow	11	2	19	60	8	Q-10	+	+
MK348	*S. viridis*	Orange	3	10	16	58	13	Q-10	+	+
MK355	*E. crus-galli*	Yellow	11	2	17	57	13	Q-10	+	+

[a] Monosaccharide-type glycosphingolipid.
[b] Oligosaccharide-type glycosphingolipid.

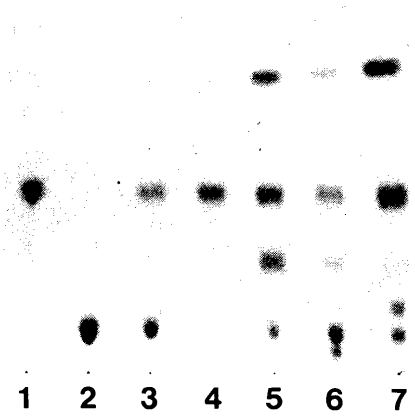

Fig. Profiles of Glycosphingolipids Extracted from the *Sphingomonas*-like Isolates.

Purified glycosphingolipids of *S. paucimobilis* IAM 12576 and glycosphingolipids of the isolated *Sphingomonas*-like strains were analyzed by TLC. Lipids were made visible by spraying 10% sulfuric acid in ethanol, and heating. Lanes 1, monosaccharide-type glycosphingolipid of IAM 12576; 2, tetrasaccharide-type glycosphingolipid of IAM 12576; 3, MK355; 4, MK329; 5, MK346; 6, MK347; 7, MK341.

Sphingomonas-type profile. Yellow-pigmented small colonies, which were often identified as *Sphingomonas*, were not easily isolated from agar plates without polymyxin B because of disturbance from fast-growing colonies. These results proved that polymyxin B was very effective to concentrate *Sphingomonas*-like strains.

For further identification of 54 *Sphingomonas*-like isolates described above, ubiquinone was extracted from 50 mg of lyophilized cells by the method of Yamada *et al.*,[9] purified by thin-layer chromatography (TLC) on silica gel (Merck), and analyzed by high-performance liquid chromatography (HPLC) using an ODS-80T_M column (Tosoh). Since glycosphingolipid is the most characteristic cellular component of *Sphingomonas* species, it was extracted by the method of Kawahara *et al.*[6] with some modifications. Lyophilized cells (50 mg) were extracted with chloroform/methanol (C/M) (2 : 1, v/v), and glycosphingolipid was extracted with C/M (1 : 3, v/v) at 80°C from the residue of the first extraction. To remove phospholipid contained in the glycosphingolipid fraction, the extract was treated with 0.1 M NaOH at 100°C for 1 h in a screw-capped glass tube, neutralized after the reaction, dialyzed, and lyophilized. The glycosphingolipid was analyzed by TLC with the solvent system described previously.[6]

All of 54 *Sphingomonas*-like isolates contained 2-hydroxymyristic acid as the sole hydroxy fatty acid, myristic acid, palmitic acid, palmitoleic acid, and *cis*-vaccenic acid as non-polar fatty acids, ubiquinone Q-10, and glycosphingolipid. The characteristics of ten isolates are summarized in Table as representatives of 54 strains. These chemotaxonomical characters indicated that all of 54 strains belonged to the genus *Sphingomonas*. As shown in Fig., monosaccharide-type glycosphingolipid occurred in all strains. Although most of the strains had a spot with an R_F value identical to the tetrasaccharide-type glycosphingolipid of *S. paucimobilis*, for example, MK355 (Fig., lane 3), variations of the TLC profile were observed in some other strains (lane 5–7). No spot for oligosaccharide-type glycosphingolipid was observed in the strain MK329 (lane 4). Further studies are required to clarify the chemical structure of the unknown glycosphingolipids contained in these *Sphingomonas* strains.

In this study we found that sphingomonads could be isolated from ears of rice and taxonomically related plants at a high frequency, but very seldom isolated from wheat and barley. When we tried to isolate sphingomonads from other organs of rice plants, the frequency of isolation was very low. These results indicated that the population of sphingomonads depends on the species and organ of plants. However, at least the ears of rice, *Echinochloa*, and *Setaria*, and probably that of more species of the family Gramineae, are the place where sphingomonads are living as members of the natural microbiological flora. As reported in our previous papers,[6,7] *S. paucimobilis* lacks lipopolysaccharide, and the outer membrane is composed of glycosphingolipid in addition to phospholipid and membrane protein. This unusual characteristic of the cell envelope of *Sphingomonas* may have some physiological meaning for growth and survival on the plant surface.

Acknowledgments. The authors are very grateful to Professor K. Komagata and Dr. H. Oyaizu for helpful discussions and suggestions. The authors also thank Mr. T. Nakazato for his kind help for this study.

References

1) E. Yabuuchi, I. Yano, H. Oyaizu, Y. Hashimoto, T. Ezaki, and H. Yamamoto, *Microbiol. Immunol.*, **34**, 99–119 (1990).
2) B. Holmes, R. J. Owen, A. Evans, H. Malnick, and W. R. Wilcox, *Int. J. Syst. Bacteriol.*, **27**, 133–146 (1977).
3) D. Thomas-Bauzon, P. Weinhard, T. Heulin, and J. Balandreau, *Can. J. Microbiol.*, **28**, 922–928 (1982).
4) R. Bally, D. Thomas-Bauzon, T. Heulin, J. Balandreau, C. Richard, and L. De Ley, *Can. J. Microbiol.*, **29**, 881–887 (1983).
5) Y. Oyaizu-Masuchi and K. Komagata, *J. Gen. Appl. Microbiol.*, **34**, 127–164 (1988).
6) K. Kawahara, U. Seydel, M. Matsuura, H. Danbara, E. Th. Rietschel, and U. Zähringer, *FEBS Lett.*, **292**, 107–110 (1991).
7) S. Kawasaki, R. Moriguchi, K. Sekiya, T. Nakai, E. Ono, K. Kume, and K. Kawahara, *J. Bacteriol.*, **176**, 284–290 (1994).
8) K. Kawahara, K. Uchida, and K. Aida, *Biochim. Biophys. Acta*, **712**, 571–575 (1982).
9) Y. Yamada, K. Aida, and T. Uemura, *J. Gen. Appl. Microbiol.*, **15**, 181–196 (1969).