Distribution of 3-Ketolactose Formation among *Sphingomonas* spp. and Other Members of the Alpha Subclass of the *Proteobacteria*

TAKESHI SAKANE,^{1*} MARIKO TAKEUCHI,¹ ANDRE DE BRUYN,²
KAREL KERSTERS,³ AND AKIRA YOKOTA^{1†}

*Institute for Fermentation, Osaka, Yodogawa-ku, Osaka 532, Japan,¹ and Laboratorium voor Organische Chemie,²
and Laboratorium voor Microbiologie,³ Rijksuniversiteit Gent, B-9000 Ghent, Belgium*

The distribution of 3-ketolactose formation among members of the alpha subclass of the *Proteobacteria* was investigated by the Benedict reagent test and by a more sensitive quantitative method in which high-performance liquid chromatography was used. 3-Ketolactose formation activity was found in strains of *Agrobacterium* biovar 1 and in strains of eight species of the genus *Sphingomonas*, including *Sphingomonas paucimobilis* (the type species), which belong to the alpha-2 and alpha-4 subclasses of the *Proteobacteria*, respectively. Weak activity was found in *Erythrobacter longus* IFO 14126^T (T = type strain), a member of the alpha-4 subclass of the *Proteobacteria*. The ketosugar was not produced by members of the other taxa of the alpha subclass of the *Proteobacteria* tested. The ketosugar was isolated from culture broths of *S. paucimobilis* IFO 13935^T and *Sphingomonas yanoikuyae* IFO 15102^T and was identified as 3-ketolactose [4-*O*-(β-D-xylo-hexopyranosyl-3-ulose)-D-glucopyranose] by chromatographic analyses and ¹H nuclear magnetic resonance spectroscopy.

Formation of 3-ketolactose (3KL) [4-*O*-(β-D-xylo-hexopyranosyl-3-ulose)-D-glucopyranose] from lactose is characteristic of strains of *Agrobacterium* biovar 1 and has been widely used as a taxonomic marker to differentiate distinct biovar groups of the genus *Agrobacterium* (3–6, 11, 13). This reaction has been reported to be catalyzed by a hexopyranoside:cytochrome *c* oxidoreductase (EC 1.1.99.13) (25). The structure of 3KL was elucidated by nuclear magnetic resonance (NMR) spectroscopy (100 MHz) and polarography in 1969 (1). Recently, the NMR spectrum of 3KL was completely analyzed at 500 MHz by two-dimensional NMR techniques (7).

Holmes and Roberts (9) found that strains of the so-called “*Agrobacterium* yellow group” are also positive in the 3KL test, although DNA-rRNA hybridization experiments revealed that these bacteria are more closely related to *Sphingomonas capsulata* than to the genus *Agrobacterium* (2, 8). During studies on strains isolated from roots of plants, we found that certain yellow-pigmented bacteria were positive in the 3KL test and that these organisms could be classified in the genus *Sphingomonas* on the basis of biochemical and chemotaxonomic characteristics.

The genus *Sphingomonas* was proposed by Yabuuchi et al. (28) for yellow-pigmented, nonfermentative, gram-negative rods. These bacteria contain large amounts of a unique sphingoglycolipid and ubiquinone 10 and have high DNA G+C contents (62 to 68 mol%). Members of the genus *Agrobacterium* are known to belong to the alpha-2 subclass of the *Proteobacteria* (21, 26), whereas members of the genus *Sphingomonas* belong to the alpha-4 subclass (24). Thus far, formation of 3KL from lactose has been thought to be a characteristic property of biovar 1 of the genus *Agrobacterium*, and this activity has not been found in any other taxon previously.

In this paper we describe the production of 3KL by members of the genus *Sphingomonas* and the distribution of 3KL-pro-

ducing activity among members of the alpha subclass of the *Proteobacteria*.

MATERIALS AND METHODS

Bacterial strains. The strains belonging to the genus *Sphingomonas* examined in this study are listed in Table 1. Three of the five strains previously classified as members of the “*Agrobacterium* yellow group” by Holmes and Roberts (9) (IFO 15208^T [T = type strain], IFO 15209, and IFO 15210) were obtained from the National Collection of Plant Pathogenic Bacteria, Harpenden, England, where they are deposited under the name [*Agrobacterium rhizogenes*], and two strains (IFO 15163 and IFO 15164) were obtained from the National Collection of Type Cultures, London, England, where they are deposited under the name [*Chromobacterium lividum*]. Three strains that were isolated from roots of rice plants and paddy soil (IFO 15495, IFO 15496, and IFO 15497) were obtained from J. Sugiyama, Institute of Molecular and Cellular Biosciences, The University of Tokyo, Tokyo, Japan; these strains were identified as *Alcaligenes* sp. strains. The origins of the 44 reference strains belonging to the alpha subclass of the *Proteobacteria* used in this study are shown in Table 2.

3KL formation from lactose. All of the bacterial strains except *Erythrobacter longus* IFO 14126^T and *Roseobacter denitrificans* IFO 15277^T were grown on a solid medium containing 1% Bacto Yeast Extract (Difco), 2% glucose, 2% calcium carbonate, and 2% agar in deionized water for 2 to 4 days at 30°C. Three loopfuls of cells were inoculated into 30 ml of YL broth (0.1% Bacto Yeast Extract and 1% lactose in deionized water; pH 6.8) and incubated with rotary shaking for 3 days at 30°C. *E. longus* IFO 14126^T and *Roseobacter denitrificans* IFO 15277^T, which were isolated from seaweed, were grown in the same media prepared with 75% seawater instead of deionized water and incubated at 24°C. After cells were removed by centrifugation, the culture broths were assayed for 3KL.

Detection of 3KL. 3KL was detected by adding 0.2 ml of the Benedict reagent to 1 ml of culture broth. After incubation for 60 min at room temperature (25 to 28°C), each mixture was examined for the formation of yellow precipitates of Cu₂O resulting from reaction with 3KL. 3KL and other sugars were identified by the method of Mikami and Ishida (16), except that compounds were eluted with 0.4 M boric acid-KOH buffer (pH 9.0); a model LC-9A high-performance liquid chromatography (HPLC) apparatus (Shimadzu) equipped with a Shim-pac ISA O7/S2504 column (250 by 4 mm) and a Shimadzu model RF 10A spectrofluorometer was used for this analysis. 3KL purified from culture broth of *Agrobacterium radiobacter* IFO 13532^T (biovar 1) was used as the standard.

Purification of 3KL. Pure 3KL was isolated from 500-ml portions of 5-day culture broths of *A. radiobacter* IFO 13532^T, *Sphingomonas paucimobilis* IFO 13935^T, and *Sphingomonas yanoikuyae* IFO 15102^T. After cells were removed, each supernatant was freeze-dried, and the dried material was dissolved in a small amount of water. Then 3 volumes of ethanol and 1 volume of a chloroform-isomyl alcohol mixture (24:1, vol/vol) were added, and the solution was centrifuged. After the supernatant was treated with IR 120(H⁺) ion exchange resin and then with IR 45(OH⁻) ion exchange resin, the filtrate was concentrated to a syrup. The ketosugar was precipitated by adding methanol and keeping the solution in a refrigerator. Recrystallization from methanol was repeated to purify the ketosugar.

* Corresponding author. Mailing address: Institute for Fermentation, Osaka, 17-85, Juso-honmachi 2-chome, Yodogawa-ku, Osaka 532, Japan. Phone: 81-6-300-6555. Fax: 81-6-300-6814.

† Present address: Institute of Molecular and Cellular Biosciences, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan.

TABLE 1. Strains of *Sphingomonas* species used

Species	IFO no. ^a	Obtained from ^b :	Source	Reference
<i>S. adhaesiva</i>	15099 ^T	JCM 7370 ^T	Water	28
<i>S. asaccharolytica</i>	15499 ^T	Yasuda, as isolate Y-345 ^T	Root of apple tree	
<i>S. capsulata</i>	12533 ^T	ATCC 14666 ^T	Water	28
<i>S. macrogoltabidus</i>	15033 ^T		Soil	22
<i>S. mali</i>	15500 ^T	Yasuda, as isolate Y-347 ^T	Root of apple tree	
	15501	Yasuda, as isolate Y-348	Root of apple tree	
	15502	Yasuda, as isolate Y-351	Root of apple tree	
<i>S. parapaucimobilis</i>	15100 ^T	JCM 7510 ^T	Urine	28
<i>S. paucimobilis</i>	13935 ^T	JCM 7516 ^T	Hospital respirator	28
	15495	Sugiyama, as <i>Alcaligenes</i> sp. strain OGS47	Paddy soil	18
	15496	Sugiyama, as <i>Alcaligenes</i> sp. strain Y-22	Paddy soil	18
	15497	Sugiyama, as <i>Alcaligenes</i> sp. strain Y-39	Root of rice plant	18
<i>S. pruni</i>	15498 ^T	Yasuda, as isolate Y-250 ^T	Root of peach tree	
<i>S. rosa</i>	15208 ^T	NCPB 2661 ^T , as [<i>A. rhizogenes</i>]	Hairy root of <i>Rosa</i> sp.	9
	15209	NCPB 2662, as [<i>A. rhizogenes</i>]	Hairy root of <i>Rosa</i> sp.	9
	15210	NCPB 2663, as [<i>A. rhizogenes</i>]	Hairy root of <i>Rosa</i> sp.	9
<i>S. sanguis</i>	13937 ^T	JCM 7514 ^T	Blood	22
<i>S. terrae</i>	15098 ^T		Soil	22
<i>S. yanoikuyae</i>	15102 ^T	JCM 7371 ^T	Clinical specimen	28
	15163	NCTC 10590, as [<i>C. lividum</i>]	<i>Psychotria nairobiensis</i>	9
	15164	NCTC 10591, as [<i>C. lividum</i>]	Tissue culture of <i>Ardisia crispa</i>	9

^a IFO, Institute for Fermentation, Osaka, Yodogawa-ku, Osaka, Japan.

^b JCM, Japan Collection of Microorganisms, RIKEN, Wako, Saitama, Japan; ATCC, American Type Culture Collection, Rockville, Md.; NCTC, National Collection of Type Cultures, London, England; NCPB, National Collection of Plant Pathogenic Bacteria, Hatching Green, Harpenden, England; Sugiyama, J. Sugiyama, Institute of Molecular and Cellular Biosciences, The University of Tokyo, Tokyo, Japan; Yasuda, M. Yasuda, Fruit Tree Research Station, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Japan.

Identification of 3KL. Thin-layer chromatography was performed by using cellulose thin layers (SPOTFILM S205; Tokyo Kasei Kogyo Co., Ltd.) and ethyl acetate-pyridine-acetic acid-water (5:5:1:3, by volume) as the developing solvent, followed by spraying with 1% triphenyltetrazolium chloride (TTC) in 0.5 N NaOH. ¹H NMR spectra of the 3KL samples (in D₂O) were obtained at 20°C with a Bruker model AM500 spectrometer operating at 500.12 MHz by using a pulse angle of 19° and a resolution of 0.33 Hz per point. These spectra were analyzed by comparing them with the data of De Bruyn and Van Beeumen (7), which were obtained from two-dimensional NMR experiments performed at 500 MHz.

RESULTS AND DISCUSSION

Formation of 3KL in *Sphingomonas* species. The results of the experiments to determine production of 3KL by the 21 *Sphingomonas* strains examined are shown in Table 3. A total of 15 strains, including 3 *S. paucimobilis* strains, 1 *Sphingomonas parapaucimobilis* strain, 3 *S. yanoikuyae* strains, 1 *Sphingomonas asaccharolytica* strain, 3 *Sphingomonas mali* strains, 1 *Sphingomonas pruni* strain, and 3 *Sphingomonas rosa* strains, were 3KL positive as determined by detection with the Benedict reagent, and 3KL concentrations of 0.15 to 2.4 mg/ml were found when the culture broths were analyzed by HPLC. Two strains, *S. paucimobilis* IFO 15497 and *Sphingomonas sanguis* IFO 13937^T, gave negative results with the Benedict reagent, but 3KL was detected at low concentrations (0.03 and 0.02 mg/ml, respectively) in the culture broths of these organisms by HPLC (Table 3). Thus, 17 strains belonging to eight *Sphingomonas* species produced 3KL from lactose. Four *Sphingomonas* strains, *Sphingomonas adhaesiva* IFO 15099^T, *S. capsulata* IFO 12533^T, *Sphingomonas macrogoltabidus* IFO 15033^T, and *Sphingomonas terrae* IFO 15098^T, were negative for 3KL production.

The eight strains isolated from plants and paddy soil and the misclassified strains [*A. rhizogenes*] IFO 15208^T, IFO 15209, and IFO 15210 and [*C. lividum*] IFO 15163 and IFO 15164, which were assigned to the *Agrobacterium* yellow group by Holmes and Roberts (9), have been found to belong to the genus *Sphingomonas* on the basis of the following biochemical

and chemotaxonomic characteristics: production of yellow pigment, presence of ubiquinone Q-10 and large amounts of sphingolipid, presence of 2-hydroxy fatty acids but not 3-hydroxy fatty acids as cellular hydroxy fatty acids, and DNA G+C contents of 64.0 to 65.9 mol%. The biochemical and chemotaxonomic characteristics of these strains are described in the accompanying paper (23). Of these 13 strains, 3 (IFO 15495, IFO 15496, and IFO 15497) were identified as *S. paucimobilis*, and 2 (IFO 15163 and IFO 15164) were identified as *S. yanoikuyae*. The remaining eight strains differed from the previously described species of the genus *Sphingomonas* and are described as *Sphingomonas rosa*, *Sphingomonas pruni*, *Sphingomonas asaccharolytica*, and *Sphingomonas mali* (Table 1) in the accompanying paper (23).

The changes in the 3KL concentrations in culture broths during cultivation of six strains of *Sphingomonas* species and *A. radiobacter* IFO 13532^T are shown in Table 4. In the culture broth of *A. radiobacter* IFO 13532^T, about 60% of the initial lactose was oxidized to 3KL after incubation for 1 day, and the concentration of the accumulated 3KL decreased slightly after 4 days of incubation. Although this strain was *o*-nitrophenyl-β-D-galactopyranoside test positive (data not shown), neither glucose nor galactose was found in the culture broth. A similar change in sugar content was found in the culture broth of *S. yanoikuyae* IFO 15163, but the amount of 3KL produced was about one-half that produced by *A. radiobacter* IFO 13532^T. Lactose was catabolized slowly by *S. rosa* IFO 15208^T, but the amount of 3KL in the culture broth increased when the incubation time was increased to 4 days. Small amounts of 3KL and large amounts of glucose and galactose accumulated in the culture broths of *S. pruni* IFO 15498^T, *S. asaccharolytica* IFO 15499^T, and *S. mali* IFO 15500^T. After incubation for 4 days, the amounts of 3KL in these cultures were 12 to 23% of the amounts found after 1 day of incubation, whereas the concentrations of glucose and galactose had increased. No 3KL was detected at any time in the culture broth of *S. capsulata* IFO 12533^T. These results indicate that during incubation 3KL pro-

TABLE 2. 3KL formation in members of the alpha subclass of the *Proteobacteria*

Species ^a	IFO no. ^b	Original designation ^c	Detection by Benedict reagent ^d	Amt of sugar (mg/ml) as determined by HPLC				
				3KL	Lactose	Glucose	Galactose	
Alpha-1 subclass								
<i>Aquaspirillum polymorphum</i>	13961 ^T	ATCC 11332 ^T	—	— ^e	9.53	0.03	0.16	
<i>Azorhizobium caulinodans</i>	14845 ^T	LMG 6465 ^T	—	—	6.55	—	0.28	
<i>Rhodospirillum rubrum</i>	3986		—	—	9.89	—	0.02	
Alpha-2 subclass								
<i>Agrobacterium radiobacter</i> (biovar 1)	13532 ^T	ATCC 19358 ^T	+++	9.09	0.19	—	—	
<i>Agrobacterium rhizogenes</i> (biovar 1)	14554	NIAES 1724	+++	8.02	0.06	0.01	0.01	
<i>Agrobacterium rhizogenes</i> (biovar 2)	13257 ^T	ATCC 11325 ^T	—	—	4.41	—	—	
<i>Agrobacterium rubi</i>	13261 ^T	ATCC 13335 ^T	—	—	5.66	0.02	—	
<i>Agrobacterium tumefaciens</i> (biovar 1)	13264	ATCC 11157	+++	6.17	2.10	—	0.01	
<i>Agrobacterium tumefaciens</i> (biovar 2)	15196	SAES R-6	—	—	9.57	—	—	
<i>Agrobacterium vitis</i> (biovar 3)	15140 ^T	NCPPB 3554 ^T	—	—	5.87	0.01	—	
<i>Beijerinckia indica</i>	3744	ATCC 9037	—	—	9.80	—	—	
<i>Bradyrhizobium japonicum</i>	14783 ^T	ATCC 10324 ^T	—	—	6.65	—	—	
<i>Brevundimonas diminuta</i>	12697 ^T	ATCC 11568 ^T	—	—	9.50	0.05	0.02	
<i>Brevundimonas vesicularis</i>	12165 ^T	ATCC 11426 ^T	—	—	8.93	—	—	
<i>Mycoplana bullata</i>	13290 ^T	ATCC 4278 ^T	—	—	10.05	—	0.03	
<i>Mycoplana dimorpha</i>	13291 ^T	ATCC 4279 ^T	—	—	10.07	—	—	
<i>Mycoplana ramosa</i>	15249 ^T	NCIB 9440 ^T	—	—	10.01	—	—	
<i>Rhizobium fredii</i>	14780 ^T	ATCC 35423 ^T	—	—	5.77	0.04	0.25	
<i>Rhizobium galegae</i>	14965 ^T	HAMBI 540 ^T	—	—	6.36	—	0.19	
<i>Rhizobium huakuii</i>	15243 ^T	CCBAU 2609 ^T	—	—	6.08	—	—	
<i>Rhizobium leguminosarum</i>	14778 ^T	ATCC 10004 ^T	—	—	4.43	—	—	
<i>Rhizobium loti</i>	14779 ^T	ATCC 33669 ^T	—	—	5.90	—	—	
<i>Rhizobium meliloti</i>	14782 ^T	ATCC 9930 ^T	—	—	2.70	3.55	3.58	
<i>Rhizobium tropici</i>	15247 ^T	CIAT 899 ^T	—	—	3.11	—	—	
<i>Thiobacillus novellus</i>	14993 ^T	ATCC 8093 ^T	—	—	10.03	—	—	
Alpha-3 subclass								
<i>Paracoccus denitrificans</i>	13301	ATCC 19367	—	—	9.89	—	—	
<i>Rhodobacter sphaeroides</i>	12203 ^T	NCIB 8253 ^T	—	—	8.38	—	0.01	
<i>Roseobacter denitrificans</i>	15277 ^T	Shiba, Och 101 ^T	—	—	5.55	—	0.49	
<i>Thiobacillus versutus</i>	14567 ^T	ATCC 25364 ^T	—	—	9.65	—	—	
Alpha-4 subclass								
<i>Blastobacter natatorius</i>	15649 ^T	ATCC 35951 ^T	—	—	6.37	0.03	0.24	
<i>Erythrobacter longus</i>	14126 ^T	Shiba, Och 101 ^T	—	tr ^f	5.92	0.01	0.43	
<i>Rhizomonas subterfaciens</i>	15211 ^T	NCPPB 3629 ^T	—	—	6.23	—	—	
Alpha subclass								
<i>Acetobacter aceti</i>	14818 ^T	NCIB 8621 ^T	—	—	10.05	—	—	
<i>Acetobacter Hansenii</i>	14820 ^T	NCIB 8746 ^T	—	—	10.08	—	—	
<i>Acetobacter liquefaciens</i>	12388 ^T	IAM 1834 ^T	—	—	9.93	—	—	
<i>Acetobacter pasteurianus</i>	13752	NCIB 8622	—	—	10.05	—	—	
<i>Aquaspirillum itersonii</i>	15648 ^T	ATCC 12639 ^T	—	—	9.96	—	0.01	
<i>Aquaspirillum peregrinum</i>	14922 ^T	NCIB 9435 ^T	—	—	9.86	0.02	0.04	
<i>Gluconobacter asaii</i>	3276 ^T		—	—	10.05	—	—	
<i>Gluconobacter cerinus</i>	3567 ^T		—	—	10.07	—	—	
<i>Gluconobacter frateurii</i>	3264 ^T		—	—	10.04	—	—	
<i>Gluconobacter oxydans</i>	14819 ^T	NCIB 9013 ^T	—	—	10.06	—	—	
<i>Xanthobacter autotrophicus</i>	14758	DSM 1618	—	—	9.74	—	0.02	
<i>Xanthobacter flavus</i>	14759 ^T	DSM 338 ^T	—	—	9.34	—	0.09	

^a Subclass assignments based on data from references 6, 10, 12, 15, 24, 27, and 29.^b IFO, Institute for Fermentation, Osaka, Yodogawa-ku, Osaka, Japan.^c ATCC, American Type Culture Collection, Rockville, Md.; LMG, Culture Collection of the Laboratorium voor Microbiologie, Rijksuniversiteit Gent, Ghent, Belgium; NIAES, National Institute of Agro-Environmental Sciences, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Japan; SAES, Shizuoka Agricultural Experiment Station, Shizuoka, Japan; NCPPB, National Collection of Plant Pathogenic Bacteria, Hatching Green, Harpenden, England; HAMBI, Culture Collection of the Department of Microbiology, University of Helsinki, Helsinki, Finland; CCBAU, Culture Collection of Beijing Agricultural University, Beijing, People's Republic of China; CIAT, Centro de Investigacion sobre Fijacion de Nitrogeno, Universidad Nacional Autonoma de Mexico, Morelos, Mexico; NCIB, National Collection of Industrial Bacteria, Torrey Research Station, Aberdeen, Scotland; Shiba, T. Shiba, Ocean Research Institute, The University of Tokyo, Iwate, Japan; IAM, Microbial and Microalgal Research Center, Institute of Applied Microbiology, The University of Tokyo, Tokyo, Japan; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.^d + + +, strongly positive; —, negative.^e —, not detected (concentration, <0.003 mg/ml).^f tr, trace (0.003 mg/ml).

duced from lactose is catabolized by *Sphingomonas* species as well as by agrobacteria (11).

Identification of 3KL produced by *Sphingomonas* species. The chemical characteristics of the ketosugars purified from

the culture broths of *S. paucimobilis* IFO 13935^T and *S. yanoikuyae* IFO 15102^T were compared with the chemical characteristics of the ketosugar produced by *A. radiobacter* IFO 13532^T. These three ketosugars exhibited the same reten-

TABLE 3. Production of 3KL and other sugars from lactose by members of the genus *Sphingomonas* and *A. radiobacter*

Strain	Detection by Benedict reagent ^a	Amt of sugar (mg/ml) as determined by HPLC ^b			
		3KL	Lactose	Glucose	Galactose
<i>S. adhaesiva</i> IFO 15099 ^T	—	— ^c	9.89	—	—
<i>S. asaccharolytica</i> IFO 15499 ^T	w	0.31	4.68	2.32	0.67
<i>S. capsulata</i> IFO 12533 ^T	—	—	1.16	0.01	0.02
<i>S. macrogoltabidus</i> IFO 15033 ^T	—	—	6.38	0.03	0.13
<i>S. mali</i> IFO 15500 ^T	w	0.26	4.55	2.37	1.07
<i>S. mali</i> IFO 15501	w	0.20	1.08	3.65	1.17
<i>S. mali</i> IFO 15502	w	0.18	5.06	1.95	0.91
<i>S. parapaucimobilis</i> IFO 15100 ^T	w	0.34	0.14	0.86	0.11
<i>S. paucimobilis</i> IFO 13935 ^T	+	1.25	0.07	2.08	0.29
<i>S. paucimobilis</i> IFO 15495	w	0.15	1.32	1.04	0.19
<i>S. paucimobilis</i> IFO 15496	w	0.18	2.93	0.73	0.43
<i>S. paucimobilis</i> IFO 15497	—	0.03	1.60	1.81	1.63
<i>S. pruni</i> IFO 15498 ^T	w	0.20	4.63	1.78	1.25
<i>S. rosa</i> IFO 15208 ^T	+	2.02	2.76	—	—
<i>S. rosa</i> IFO 15209	+	0.86	4.58	—	0.05
<i>S. rosa</i> IFO 15210	+	0.80	7.34	0.02	—
<i>S. sanguis</i> IFO 13937 ^T	—	0.02	1.12	2.31	0.34
<i>S. terrae</i> IFO 15098 ^T	—	—	6.12	—	—
<i>S. yanoikuyae</i> IFO 15102 ^T	+	2.40	4.36	0.07	0.02
<i>S. yanoikuyae</i> IFO 15163	+	2.02	3.53	0.16	0.14
<i>S. yanoikuyae</i> IFO 15164	+	2.32	3.63	0.17	0.17
<i>A. radiobacter</i> IFO 13532 ^T	+++	5.94	0.12	—	—

^a +++, strongly positive; +, moderately positive; w, weakly positive; —, negative.^b Small amounts of an unknown sugar(s) were also detected.^c —, not detected (concentration, <0.003 mg/ml).

tion time (39.5 min) during HPLC and the same migration distance (R_f , 0.28) on thin-layer chromatography preparations. All of the sugars gave positive reactions in the Benedict reagent and alkaline-TTC tests. The chemical shifts of the 3KL samples in D₂O solutions were very close to the reference values (7) (Table 5), confirming the chemical structure of these compounds as 3KL. When 3KL is crystallized from methanol, it exists as a hemiacetal with the OCH₃ group equatorial (7).

Distribution of 3KL formation among members of the alpha subclass of the *Proteobacteria*. Table 2 summarizes the results of the Benedict reagent test and the sugar compositions (as determined by HPLC) of the culture broths of 44 strains belonging to the alpha subclass of the *Proteobacteria*. The alpha subclass has been divided into four subgroups, the alpha-1, alpha-2, alpha-3, and alpha-4 subgroups (27). 3KL was detected only in some members of the alpha-2 and alpha-4 subclasses. None of the members of the alpha-1 and alpha-3 subclasses investigated formed 3KL.

In the alpha-2 subclass, 3KL was produced by *Agrobacterium* biovar 1 strains *A. radiobacter* IFO 13532^T, *A. rhizogenes* IFO

14554, and *Agrobacterium tumefaciens* IFO 13264, but 3KL was not found in the culture broths of other agrobacteria, including *A. rhizogenes* IFO 13257^T (biovar 2), *A. tumefaciens* IFO 15196 (biovar 2), *Agrobacterium vitis* IFO 15140^T (previously classified as a member of biovar 3) (17), and *Agrobacterium rubi* IFO 13261^T. Furthermore, the test for production of 3KL from lactose was negative with the following taxa belonging to the alpha-2 subclass: *Beijerinckia indica*, *Bradyrhizobium japonicum*, three species of the genus *Mycoplana*, two species of the genus *Brevundimonas* (previously assigned to the taxa *Pseudomonas diminuta* and *Pseudomonas vesicularis*) (20), seven species of the genus *Rhizobium*, and *Thiobacillus novellus*. *E. longus* IFO 14126^T, a member of the alpha-4 subclass, was weakly 3KL positive; although the culture broth of this strain exhibited a negative reaction in the Benedict reagent test, a trace of 3KL (0.003 mg/ml) was found in the culture broth. *Blastobacter natatorius* IFO 15649^T and *Rhizomonas suberifaciens* IFO 15211^T, which are also members of the alpha-4 subclass (10, 24), did not produce 3KL.

In conclusion, the 3KL-producing bacteria belonging to the

TABLE 4. Changes in sugar contents of lactose-containing culture broths during cultivation of *Sphingomonas* and *A. radiobacter* strains

Strain	Amt of sugar (mg/ml) as determined by HPLC after:							
	1 day				4 days			
	3KL	Lactose	Glucose	Galactose	3KL	Lactose	Glucose	Galactose
<i>S. yanoikuyae</i> IFO 15163	3.02	1.88	— ^a	—	2.16	—	—	—
<i>S. rosa</i> IFO 15208 ^T	1.44	5.80	—	—	1.88	1.64	—	—
<i>S. pruni</i> IFO 15498 ^T	0.66	4.38	0.60	0.28	0.08	0.09	2.83	1.22
<i>S. asaccharolytica</i> IFO 15499 ^T	0.88	4.63	1.68	0.37	0.16	0.72	4.03	0.95
<i>S. mali</i> IFO 15500 ^T	0.35	5.13	1.42	0.53	0.08	0.82	3.92	0.94
<i>S. capsulata</i> IFO 12533 ^T	—	3.67	0.02	0.05	—	0.35	—	—
<i>A. radiobacter</i> IFO 13532 ^T	5.98	1.11	—	—	5.58	0.50	—	—

^a —, not detected (concentration, <0.003 mg/ml).

TABLE 5. ¹H NMR chemical shifts of 3KL in D₂O

Moiety	Reference value (ppm) ^a	Chemical shift (ppm) with 3KL formed by:		
		<i>A. radiobacter</i> IFO 13532 ^T	<i>S. paucimobilis</i> IFO 13935 ^T	<i>S. yanoikuyae</i> IFO 15102 ^T
β-D-xylo-Hexopyranosyl-3-ulose moieties				
H-1'	4.57	4.54	4.54	4.54
H-2'	3.61	3.57	3.57	3.57
H-4'	3.66	3.63	3.64	3.64
H-5'	3.99	3.97	3.97	3.97
H-6'A	3.78	3.75	3.75	3.76
H-6'B	3.78	3.75	3.75	3.76
OCH ₃ (acetal)	3.37	3.35	3.35	3.35
α-D-Glucopyranosyl moieties				
H-1	5.25	5.22	5.21	5.21
H-2	3.60	3.57	3.57	3.57
H-3	3.85	3.83	3.83	3.84
H-4	3.67	3.66	3.65	3.65
H-5	3.96	3.93	3.94	3.93
H-6A	3.90	3.87	3.88	3.88
H-6B	3.90	3.87	3.88	3.88
β-D-Glucopyranosyl moieties				
H-1	4.68	4.65	4.66	4.66
H-2	3.31	3.28	3.28	3.28
H-3	3.67	3.65	3.67	3.67
H-4	3.67	3.65	3.67	3.67
H-5	3.64	3.60	3.60	3.60
H-6A	3.97	3.95	3.94	3.95
H-6B	3.84	3.82	3.83	3.83

^a Data from reference 7.

alpha subclass of the *Proteobacteria* were members of the alpha-2 and alpha-4 subclasses. The 3KL-positive bacteria belonging to the alpha-2 subclass were restricted to strains of *Agrobacterium* biovar 1, as reported previously (6, 14). The 3KL-positive members of the alpha-4 subclass were members of eight species of the genus *Sphingomonas*, including *S. paucimobilis* (the type species), and *E. longus*. On the basis of 16S rRNA gene sequences, Takeuchi et al. have suggested that the genus *Sphingomonas* is a heterogeneous taxon that can be divided into several subgroups (24). The 3KL-positive species of the genus *Sphingomonas* were distributed among some of the subgroups of Takeuchi et al. (24). It seems that the erratic distribution of 3KL formation is due to horizontal gene transfer or plasmid-mediated gene transfer. However, the relationship between 3KL production and the taxonomic positions of bacteria is not yet clear. Furthermore, although 3KL-positive bacteria have been isolated from various sources, including plants, soil, and clinical specimens (19, 27), little information about the physical role of the enzyme in these organisms is available.

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