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IDENTIFICATION OF STRAINS ISOLATED IN THAILAND AND ASSIGNED TO THE GENERA *KOZAKIA* AND *SWAMINATHANIA*

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Summary

Four isolates, isolated from fruit of sapodilla collected at Chantaburi and designated as CT8-1 and CT8-2, and isolated from seeds of ixora („khem" in Thai, Ixora species) collected at Rayong and designated as SI15-1 and SI15-2, were examined taxonomically. The four isolates were selected from a total of 181 isolated acetic acid bacteria. Isolates CT8-1 and CT8-2 were non motile and produced a levan-like mucous polysaccharide from sucrose or D-fructose, but did not produce a water-soluble brown pigment from D-glucose on $CaCO_3$-containing agar slants. The isolates produced acetic acid from ethanol and oxidized acetate and lactate to carbon dioxide and water, but the intensity of the acetate and lactate oxidation was weak. Their growth was not inhibited by 0.35 % acetic acid (v/v) at pH 3.5. The isolates did not grow on 30 % D-glucose (w/v), and utilization of methanol was not found. Isolates SI15-1 and SI15-2 had peritrichous flagella and grew in the presence of either 0.35 % acetic acid (v/v) at pH 3.5, 3 % NaCl (w/v), or 1 % KNO_3 (w/v). Acetate and lactate were oxidized to carbon dioxide and water, but the intensity was weak. The isolates grew on mannitol agar and glutamate agar as well as on 30 % D-glucose (w/v), but did not utilize methanol. The 16S rRNA gene sequence analysis and DNA-DNA hybridization indicated that isolates CT8-1 and CT8-2 and isolates SI15-1 and SI15-2 were unequivocally identified respectively as Kozakia baliensis and Swaminathania salitolerans.

Key words: *acetic acid bacteria, Alphaproteobacteria, Kozakia baliensis, Swaminathania salitolerans, 16S rRNA gene sequences*.

Abbreviations: NITE Biological Resource Center (NBRC), Department of Biotechnology, National Institute of Technology and Evaluation (NITE), Kisarazu, Chiba, Japan; BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Pathumthani, Thailand; LMG, Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium.

Introduction

In the acetic acid bacteria that are accommodated to the *Alphaproteobacteria*, eleven genera were reported: *Acetobacter* [3], *Gluconobacter* [1], *Acidomonas* [26], *Gluconacetobacter* [30], *Asaia* [31], *Kozakia* [14], *Swaminathania* [15], *Saccharibacter* [10], *Neoasaia* [35], *Granulibacter* [8], and *Tanticharoenia* [37, 38] ([8, 10, 12, 15, 20, 34, 35, 37, 38]). Of the eleven genera reported, the genera *Kozakia* and *Swaminathania* are monotypic genera, along with the genera *Acidomonas*, *Saccharibacter*, *Neoasaia*, *Granulibacter*, and *Tanticharoenia,* and include only the type species within the genera. In addition, no additional strains have yet been reported taxonomically since the proposals of the two genera [32].

According to Lisdiyanti et al. [14], the type strain of *Kozakia baliensis* has a phenotypic characteristic of producing a mucous polysaccharide, when grown on sucrose. On the other hand, the type strain of *Swaminathania salitolerans* has a unique characteristic of showing growth in the presence of either 3 % NaCl or 1 % KNO_3 [15].

This paper aims to identify strains isolated in Thailand and assigned to the genera *Kozakia* and *Swaminathania*, the extremely rare acetic acid bacteria taxonomically and ecologically.

Materials and Methods

Isolation of acetic acid bacteria and reference strains. Four isolates designated as CT8-1 and CT8-2, which were isolated from fruit of sapodilla collected at Chantaburi on July 6, 2006, and as SI15-1 and SI15-2, which were isolated from seeds of ixora ('khem' in Thai, *Ixora* species) collected at Rayong on July 3, 2006, by an enrichment culture approach using a glucose/ethanol/yeast extract (GEY) medium. An isolation source was incubated at pH 4.5 and 30°C for 3-5 days in a liquid medium (15 ml/tube) composed of 2.0 % D-glucose, 5.0 % ethanol and 1.0 % yeast extract (all by w/v). When microbial growth was seen, the culture was streaked onto a GEY-agar plate containing 0.3 % $CaCO_3$ (w/v) [29]. Acetic acid bacteria were selected as acid-producing bacterial strains, which formed clear zones around colonies on the agar plate. *K. baliensis* NBRC 16664^T and *S. salitolerans* LMG 21291^T were used as reference strains.

Phenotypic characterization. Phenotypic characterization was carried out by incubating isolates at 30 °C and pH 6.8 for two days on glucose/yeast extract/peptone/glycerol (GYPG) agar, which was composed of 1.0 % D-glucose, 1.0 % glycerol, 0.5 % yeast extract, 1.0 % peptone, and 1.5 % agar (all by w/v), unless otherwise mentioned. For Gram staining of bacterial cells, the Hucker-Conn modified method was used [9]. Physiological and biochemical characterizations were made by the methods of Asai et al. [2], Yamada et al. [29], and Gosselé et al. [7]. Isolates were grown in GYPG broths containing 10 % potato extract on a rotary shaker (150-200 rpm) at 30 °C for 24 h [18].

Chemotaxonomic characterization. Acetic acid bacteria were grown in GYPG broths containing 10 % potato extract on a rotary shaker (150-200 rpm) at 30 °C for 24 h [18]. Ubiquinone was extracted from freeze-dried cells by shaking with a mixture of chloroform-methanol (2:1, v/v). Cells were removed by filtration, and the combined filtrates were evaporated to dryness under a reduced pressure on a rotary evaporator. The resulting residue was dissolved in a small volume of acetone, followed by thin-layer chromatography on a silica gel plate (20x 20 cm, silica gel $60F_{254}$, Art 5715, E. Merck, Darmstdt, Germany) with a solvent system of pure benzene [28]. The purified ubiquinone preparation was applied to reversed-phase paper chromatography [28, 29] and to high performance liquid chromatography [21] for its homologues.

Cellular fatty acid composition. Cellular fatty acid composition was determined in cells grown on trypticase soy agar (without NaCl) for 48 h at 30 °C. Methyl esters of cellular fatty acids were prepared and identified by the instructions of the Microbial Identification system (MIDI, Hewlett Packard, Palo Alto, CA, USA).

16S rRNA gene sequencing. Bacterial 16S rRNA gene were amplified by PCR with *Taq* DNA polymerase and primers 20F (5'-GAGTTTGATCCTGGCTCAG'-3, the *Escherichia coli* numbering system [4]) and 1500R (5'GTTACCTTGTTACGACTT'-3) and sequenced, as described previously [24]. The 16S rRNA gene sequencing was carried out with an ABI PRISM BigDye Terminator v3.1 Cycle sequencing kit on an ABI PRISM model 310 Genetic Analyzer (Applied Biosystems, Foster, CA, USA). The following six primers were used for 16S rRNA gene sequencing; 20F, 1500R, 520F (5'-CAG-CAGCCGCGGTAATAC-3', positions 519-536), 520R (5'-GTATTACCGCGGCTG-

CTG-3', positions 536-519), 920F (5'AAACTC-AAATGAATT-GACGG-3', positions 907-926), and 920R (5'-CCGTCAATTCAT-TTGAGTTT-3', positions 926-907).

Sequence analyses. Multiple sequence alignments were performed for ca. 1372 bases with a program CLUSTAL X (version 1.83) [25]. Gaps and ambiguous bases were eliminated from calculation. Distance matrices for the aligned sequences were calculated by the two-parameter method of Kimura [13]. A phylogenetic tree based on 16S rRNA gene sequences was constructed by the neighbor-joining method of Saitou and Nei [19] with the program MEGA (version 4.0; [23]). The confidence values of individual branches in the phylogenetic tree were determined by using the bootstrap analysis of Felsenstein [6] based on 1,000 replications. Pair-wise 16S rRNA gene sequence similarities were calculated for 1470 bases between phylogenetically related strains.

DNA-DNA hybridization. Bacterial DNAs were prepared, as described previously [24]. DNA base composition was determined by the method of Tamaoka and Komagata [22]. DNA-DNA hybridization was carried out by the photobiotinlabeling method using microplate wells, as described by Ezaki et al. [5]. Levels of DNA-DNA hybridization were determined colorimetrically [27]. The color intensity was measured at A_{450} on a model VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Base sequence deposition numbers. All the 16S rRNA gene sequences determined here were deposited in the DDBJ database under the accession numbers, AB308056, AB368298, AB308059, and AB363646 respectively for isolates CT8-1, CT8-2, SI15-1 and SI15-2.

Results and Discussion

The four isolates, CT8-1, CT8-2, SI15-1, and SI15-2 were Gram-negative, aerobic rods, produced catalase but not oxidase, and showed clear zones on GEY/$CaCO_3$ agar plates and growth at pH 3.5 among a total of 181 isolated acetic acid bacteria. A major isoprenoid quinone of the four isolates was Q-10.

Isolates CT8-1 and CT8-2 were non motile and produced a levan-like mucous polysaccharide from sucrose or D-fructose, but did not produce a water-soluble brown pigment from D-glucose on $CaCO_3$-containing agar slants (Table 1). The two isolates produced acetic acid from ethanol and oxidized acetate and lactate to carbon dioxide and water, but the intensity of the acetate and lactate oxidation was weak. Their growth was not inhibited by 0.35 % acetic acid (v/v) at pH 3.5. Growth was shown on mannitol agar, but not on glutamate agar.

The two isolates did not grow on 30 % D-glucose (w/v), and utilization of methanol was not found as a sole source of carbon. The isolates produced dihydroxyacetone from glycerol and D-gluconate, 2-keto-D-gluconate and 5-keto-D-gluconate from D-glucose, but not 2,5-diketo-D-gluconate. Acid was produced from D-glucose, D-mannose, D-galactose, D-xylose, L-arabinose, *meso*-erythritol, glycerol, melibiose, raffinose, or ethanol, but not from L-rhamnose, D-fructose, L-sorbose, D-mannitol, D-sorbitol, dulcitol, or lactose.

The predominant fatty acid of isolates CT8-1 and CT8-2 was the straight-chain unsaturated acid of C18:1 ω7c, which amounted respectively to 66.0 and 61.7 %, in contrast to 61.8 % in *Kozakia baliensis* NBRC 16664^T (Table 2).

In a phylogenetic tree based on 16S rRNA gene sequences, the two isolates constituted a cluster along with the type strain of *K. baliensis* (Fig. 1). Isolate CT8-1 showed 99.7 % sequence similarity to *K. baliensis* NBRC 16664^T. Between the two isolates, the calculated sequence similarity was 100 %. The two isolates, CT8-1 and CT8-2 had DNA G+C contents respectively of 59.8 and 60.2 mol % (Table 1). When an isolated, single-stranded, and labeled DNA from isolate CT8-1 was hybridized with DNAs from isolates CT8-1, CT8-2, SI15-1, and SI15-2 and the type strains of *K. baliensis* and *S. salitolerans*, the calculated DNA-DNA similarities were respectively 100, 93, 28, 32, 90, and 25 %.

The data obtained above indicate that the two isolates, CT8-1 and CT8-2 were identified as *K. baliensis*, the type species of the genus *Kozakia*.

Isolates SI15-1 and SI15-2 were motile with peritrichous flagella, and produced a watersoluble brown pigment from D-glucose on $CaCO_3$-containing agar slants and acetic acid from ethanol (Table 1). The two isolates grew in the presence of either 0.35% acetic acid (v/v) at pH 3.5, 3 % NaCl (w/v) or 1 % KNO_3 (w/v). The isolates were able to assimilate 1 % KNO_3 (w/v) as a nitrogen source. Acetate and lactate were oxidized to carbon dioxide and water, but the intensity was weak. The isolates grew on mannitol agar and glutamate agar as well as on 30 % D-glucose (w/v), but did not utilize methanol. The isolates produced dihydroxyacetone from glycerol and D-gluco-

nate, 2-keto-D-gluconate and 5-keto-D-gluconate from D-glucose. Acid was produced from D-glucose, D-mannose, D-galactose, L-arabinose, L-sorbitol, glycerol, and ethanol, but not from L-rhamnose and D-mannitol.

The predominant fatty acid of isolates SI15-1 and SI15-2 was the straight-chain unsaturated acid of C18:1 ω7c, which amounted respectively to 69.6 and 59.7 %, in contrast to 67.5 % in *S. salitolerans* LMG 21291^T (Table 2).

In a phylogenetic tree based on 16S rRNA gene sequences, the two isolates constituted a cluster, along with the type strain of *S. salitolerans* LMG 21291^T (Fig. 1). Isolate SI15-1 showed 99.6 % sequence similarity to the type strain of *S. salitolerans*. Between the two isolates, the calculated sequence similarity was 100 %. The two isolates, SI15-1 and SI15-2 had DNA G+C contents respectively of 60.8 and 62.4 mol % (Table 1). When an isolated, single-stranded, and labeled DNA from isolate S115-1 was hybridized with DNAs from isolates CT8-1, CT8-2, SI15-1, and SI15-2 and the type strains of *K. baliensis* and *S. salitolerans*, the calculated DNA-DNA similarities were respectively 26, 35, 100, 90, 25, and 92 %.

Table 1. Phenotypic characteristics of isolates CT8-1, CT8-2, SI15-1, and SI15-2 assigned to the genera *Kozakia* and *Swaminathania*.

Characteristics	1	2	3	4	5	6
Production of water-soluble brown pigment(s)	-	-	-	+	+	+
Production of mucous substance(s) from sucrose	+	+	+	-	-	-
Oxidation of						
Acetate	w	w	w	w	w	w
Lactate	w	w	w	w	w	w
Production of acetic acid from ethanol	+	+	+	+	+	+
Growth in the presence of						
0.35 % acetic acid (v/v) at pH 3.5	+	+	+	+	+	+
1 % KNO_3	-	-	-	+	+	+
Growth on						
30 % D-Glucose (w/v)	w	w	w	+	+	+
Mannitol agar	+	+	+	+	+	+
Glutamate agar	-	-	-	+	+	+
Utilization of methanol	-	-	-	-	-	-
Production of dihydroxyacetone from glycerol	w	w	w	+	+	+
Acid production from						
L-Arabinose	+	+	+	+	+	+
D-Mannitol	-	-	-	-	-	-
D-Xylose	+	+	+	+	+	+
L-Rhamnose	-	-	-	-	-	-
D-Fructose	-	-	-	-	-	-
D-Mannitol	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-
Major ubiquinone	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10
G+C content (mol %)	59.8	60.2	57.2[a]	60.8	62.4	57.6-59.9[b]

Abbreviations : 1, isolate CT8-1; 2, isolate CT8-2; 3, *Kozakia baliensis* NBRC 16664^T; 4, isolate SI15-1; 5, isolate SI15-2; 6, *Swaminathania salitolerans* LMG 21291^T; +, positive; -, negative; w, weak positive; +/-, positive or negative ; [a], cited from Lisdiyanti et al. [14]; [b], cited from Loganathan and Nair [15].

Table 2. Fatty acid composition of isolates CT8-1, CT8-2, SI15-1, and SI15-2 assigned to the genera *Kozakia* and *Swaminathania*.

Fatty acids	1	2	3	4	5	6
Straight-chain fatty acid						
C14:0	0.8	1.0	1.2	0.6	1.4	0.8
C16:0	12.7	19.1	16.1	11.5	14.8	11.9
C17:0	1.3	0.6		1.4	0.5	0.7
C18:0	2.2	3.2	3.2	2.1	1.0	4.6
Unsaturated fatty acid						
C13:1 AT 12–13	1.0	0.7	0.9	1.5	3.7	1.7
C17:1 ω6c	0.7			0.9	0.7	0.6
C18:1 ω9c						0.8
C18:1 ω7c	66.0	61.7	61.8	59.7	69.6	67.5
C19:0 cyclo ω8c	2.0	1.9	1.8	10.6	4.1	4.1
Hydroxy fatty acid						
C14:0 2-OH	3.6	4.0	4.2	2.3		0.8
C16:0 2-OH	4.3	4.9	5.5	2.4		0.6
C16:0 3-OH	1.1	1.2	1.7	1.1		0.6
C18:0 3-OH			0.7			
Summed feature*						
2	1.2	1.1	1.4	1.8	2.0	0.7
Unknown fatty acid						
14.959	0.8	0.6	0.8	0.8	1.3	1.1

Only fatty acids that represent more than 0.5 % of the total fatty acids are indicated. t, trace (< 0.5 %). Abbreviations; 1, isolate CT8-1; 2, isolate CT8-2; 3, *Kozakia baliensis* NBRC 16664^T; 4, isolate SI15-1; 5, isolate SI15-2; 6, *Swaminathania salitolerans* LMG 21291^T.
*Summed feature represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 2, alde-C12:0 and/or C14:0 3-OH and/or iso I-C16:1.

The data obtained above indicate that the two isolates, SI15-1 and SI15-2 were identified as *S. salitolerans*, the type species of the genus *Swaminathania*.

The monotypic genus *Kozakia* was characterized especially by producing a levan-like polysaccharide [14]. The two isolates described here showed the same phenotypic feature as the type strain of *K. baliensis*. However, the isolation sources of the two isolates were quite different from those of the four strains isolated in Indonesia [14]. The former was from fruit of sapodilla collected in Thailand however, the latter was either palm brown sugar or ragi (starter) collected in Bali or Java, Indonesia. Accordingly, it is of interest that the two strains identified as *K. baliensis* were isolated from sapodilla, a kind of fruit other than materials such as palm brown sugar and ragi.

The monotypic genus *Swaminathania* was characterized by producing a water-soluble brown pigment on GYC agar medium [15]. The two isolates described above showed the same phenotypic features as the type strain of *S. salitolerans*. However, the isolation sources of the two isolates SI15-1 and SI15-2 were quite different from those of the two strains isolated at Pichavaram, Tamil Nadu, India [15]. The former was from ixora collected in Thailand, however, the latter was mangrove-associated wild rice collected at Pichavaram, Tamil Nadu, India. Accordingly, it is of interest that the two strains identified as *S. salitolerans* were isolated from seeds of ixora, a kind of flowers other than materials such as mangrove-associated wild rice.

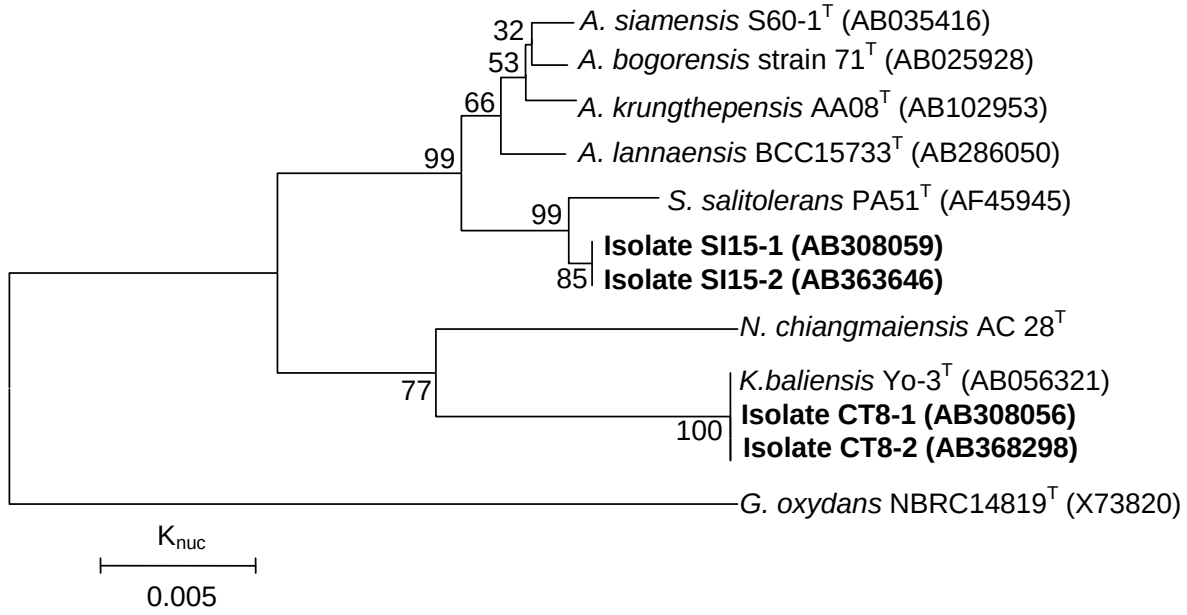


Fig. 1. Phylogenetic relationships of isolates CT8-1, C8-2, SI15-1, and SI15-2 assigned to the genera *Kozakia* and *Swaminathania*. The phylogenetic tree based on 16S rRNA gene sequences was constructed by the neighbor-joining method. The type strain of *Gluconobacter oxydans* was used as an outgroup. Numerals at the nodes indicate bootstrap percentages derived from 1,000 replications.

Upon the proposal of the genus *Swaminathania* [15], it has been indicated that the genus is closely related phylogenetically to the genus *Asaia*. The 16S rRNA gene sequence similarity was calculated to be 98.6% between the two genera. However, the former was quite different from the latter on the basis of the biochemical characteristics. The former was characterized by production of acetic acid from ethanol and growth in the presence of 0.35 % acetic acid (v/v), 3 % NaCl, and 1 % KNO_3, whereas the latter was not.

In contrast to the single species of the genus *Swaminathania*, four species are presently described in the genus *Asaia* [11, 16, 17, 31, 36]. All the four species, i.e., *Asaia bogorenis* [31], *A. siamensis* [11], *A. krungthepensis* [33], and *A. lannensis* [16, 17], actually did not show any growth in the presence of 0.35 % acetic acid (v/v), 3 % NaCl (w/v), and 1.0 % KNO_3 (w/v) and produce barely if any acetic acid.

According to Yukphan et al. [35] and Malimas et al. [16], the type strain of *S. salitolerans* was included within the *Asaia* cluster comprised of the type strains of the four *Asaia* species in phyogenetic trees based on 23S rRNA gene sequences. Considering the unique biochemical characteristics described by Loganathan and Nair [15], however, it might be adequate that the name of *Swaminathania* is retained as a separate genus.

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ИДЕНТИФИКАЦИЯ НА ЩАМОВЕ, ИЗОЛИРАНИ В ТАЙЛАНД И ОТНЕСЕНИ КЪМ РОДОВЕТЕ *KOZAKIA* И *SWAMINATHANIA*

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Разюме

Изследвани са таксономично четири щама, изолирани съответно от плод на саподила в Чантабури, означени като CT8-1 и CT8-2, и от семена на иксора („кем" на тайвански) в Районг и означени като SI15-1 и SI15-2. Четирите щама са подбрани от общо 181 изолата на оцетнокисели бактерии. Щамове CT8-1 и CT8-2 са неподвижни и продуцират леваноподобен мукополизахарид от захароза или D-фруктоза, но не отделят водноразтворим кафяв пигмент от D-глюкоза върху скосен агар, съдържащ $CaCO_3$. Изолатите произвеждат оцетна киселина от етанол и окисляват ацетат и лактат до въглероден двуокис и вода, но интензивността на окислението е слаба. Техният растеж не се инхибира от 0.35 % оцетна киселина (v/v) при pH 3.5. Изолатите не растат върху 30 % D-глюкоза (w/v), а усвояване на метанол не се установява. Щамове SI15-1 и SI15-2 имат перитрихално разположени ресни и растат в присъствие както на 0.35 % оцетна киселина (v/v) при pH 3.5, така и на 3 % NaCl (w/v) или 1 % KNO_3 (w/v). Ацетатът и лактатът се окисляват до въглероден двуокис и вода, но интензивността е слаба. Изолатите растат върху агар с манитол или глутамат, както и върху 30 % D-глюкоза (w/v), но не усвояват метанол. Проведените секвенционен анализ на 16S рРНК и ДНК-ДНК хибридизация показват, че щамове CT8-1 и CT8-2 без съмнение се идентифицират като Kozakia baliensis, а изолати SI15-1 and SI15-2 като Swaminathania salitolerans.