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Characteristics of Several Bacterial Isolates Capable of Degrading Chloroaliphatic Compounds via Hydrolytic Dechlorination

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Haloaliphatic hydrocarbons have been widely used as solvents and ingredients of pesticides and herbicides. However, when these compounds contaminate the environment, they can be very hazardous to animals and humans because of their potential toxicity and carcinogenicity. Therefore, lots of studies have been made for microbial degradation of those pollutant chemicals. In this study, 11 bacterial strains capable of degrading 1,2-dichloroethane (1,2-DCA), 2-chloropropionic acid (2-CPA), 2,3-dichloropropionic acid (2,3-DCPA), and 2-monochloroacetic acid (2-MCA) by hydrolytic dechlorination under aerobic conditions were isolated from wastewaters and rice paddy soil samples. Their morphological and biochemical characteristics and their degradation capabilities of haloaliphatic hydrocarbons were examined. On the basis of the 16S rDNA sequences, 8 different kinds of microbial species, including *Pseudomonas plecoglossicida, Xanthobacter flavus, Ralstonia eutropha,* were identified. All of the isolated strains can degrade MCA. In particular, strains UE-2 and UE-15 degraded 1,2-DCA, and strain CA-11 degraded 2,3-DCPA, which are hardly degraded by other strains.

Key words: Haloaliphatic hydrocarbons, hydrolytic dechlorination, *Xanthobacter flavus, Ralstonia eutropha*

Chemical industries produce large amounts of short-chain halogenated aliphatic hydrocarbons, which are used as organic solvents, degreasing agents, paints, pesticides, herbicides, and intermediates for the synthesis of other organic compounds. As is the case with so many industrial chemicals, haloalkanes have caused numerous cases of environmental pollution due to improper disposal of wastes, accidental spillage, or deliberate release. Such contaminations of soil, underground waters, and surface waters were frequently observed (McConnell *et al.*, 1975; Morgan and Watkinson, 1989). They caused considerable environmental pollution as well as human health problems because of their persistence and toxicity (Mohn and Tiedje, 1992; Leisinger, 1996; Lee *et al.*, 2002).

Several bacteria are known to be able to degrade these haloaliphatic pollutants, and many of these microorganisms have been isolated from the contaminated sites. From these bacterial sources, a number of enzymes involved in the degradation of halogenated compounds have been purified and characterized (Van Der Ploeg *et al.*, 1991; Bader and Leisinger, 1994; Tsang and Sam, 1999; Magnuson *et al.*, 2000). Also nucleotide sequences coding for dehalogenase have been cloned and sequenced from several bacteria (Janssen *et al.*, 1989; Schneider *et al.*, 1991; Nardi-Dei *et al.*, 1994; Maymó-Gatell *et al.*, 1999).

Dehalogenation is the critical step in the degradation of chlorinated aliphatics; because the reaction occurs as the first step in the degradative pathway without any regard to the mechanisms of hydrolytic, oxygenolytic, or reductive dehalogenation (Fetzner and Lingens, 1994; Leisinger, 1996; Janssen *et al.*, 2001). During the hydrolytic dehalogenation step of 1,2-dichloroethane (1,2-DCA), 2-chloroacetaldehyde, and 2-monochloroacetic acid (2-MCA), the chlorine substituent, which is usually responsible for the toxic and xenobiotic character of the compound, is replaced by a hydroxyl group originated from a water molecule under aerobic conditions as seen in Fig. 1.

The isolation of the bacteria capable of degrading halogenated aliphatic hydrocarbons was first carried out for the bioremediation of the contaminated chemicals. A number of soil bacteria, which synthesize dehalogenase, was found to be able to utilize halogenated alkanoic acids (Hardman *et al.*, 1986; Shin *et al.*, 2003). Olaniran *et al.* (2001) isolated 4 bacterial species from a sewage oxidation pond using the enrichment culture technique that utilized 2-monochloroacetic acid (2-MCA), $CHCl_3$, and CCl_4 for growth up to 1 g of the substrate. Hardman *et al.* (1986)

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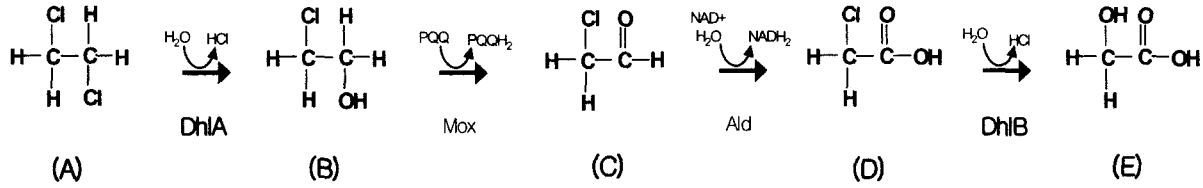

Fig. 1. Proposed catabolic pathway of 1,2-DCA and MCA under aerobic condition. (A) 1,2-dichloroethane (1,2-DCA) (B) 2-chloroethanol (C) 2-chloroacetaldehyde (D) 2-monochloroacetic acid (2-MCA) (E) glycolic acid. DhlA, haloalkane dehalogenase; Mox, alcohol dehydrogenase; Ald, aldehyde dehydrogenase; DhlB, haloacetate dehalogenase.

isolated four *Pseudomonas* species and two *Alcaligenes* species from the soil that were able to grow on halogenated alkanoic acid. They contain a large plasmid associated with the ability to utilize 2-monochloropropionic acid and 2-monochloroacetic acid and also associated with resistance towards one or more heavy metals of mercury, selenium, and aluminium. *Alcaligenes* and *Nocardia* were also isolated as trichloroethylene (TCE) degraders for the bioremediation of the soils contaminated with various chlorinated alkenes (Lee *et al.*, 2002).

In this study, 11 strains were isolated from the natural environment by enrichment cultivation on 1,2-dichloroethane (1,2-DCA), 2-chloropropionic acid (2-CPA), 2,3-dichloropropionic acid (2,3-DCPA) and 2-monochloroacetic acid (2-MCA). They were confirmed to be able to degrade those chloroaliphatics by hydrolytic dechlorination under aerobic conditions. Also, they were identified on the basis of the 16S rDNA sequence, and several isolates were further examined for their morphological and biochemical characteristics.

Materials and Methods

Isolation and cultivation of isolates

Ten grams of the samples taken from the wastewaters of Ulsan and Choengju industrial complexes and rice paddy soil were added to minimal medium MM2 ($FeSO_4 \cdot 7H_2O$, 1 μM; $(NH_4)_2SO_4 \cdot 7H_2O$, 1 mM; $CaCl_2 \cdot 2H_2O$, 100 μM; NaCl, 8.5 mM; 10 mM phosphate buffer, pH 7.0) in 400 ml flasks with each containing haloalipohatic hydrocarbons as a sole carbon and energy source. 0.5 mM of 1,2-dichloroethane (1,2-DCA), 2-chloropropionic acid (2-CPA), 2,3-dichloropropionic acid (2,3-DCPA) and 1 mM of 2-monochloroacetic acid (2-MCA) were added as a substrate after pH adjustment, according to the method described according to Hasan *et al.* (1994). These culture were cultivated in a shaking incubator at 30°C for 72 h under aerobic conditions. After enrichment in the same broth media twice, single colonies were isolated from the agar medium containing the same ingredient. The isolates were examined for the hydrolytic dechlorination of each chloroaliphatic compound.

Hydrolytic dechlorination of chloroaliphatics

After the 16 h cultivation under aerobic conditions, isolated strains were washed with 10 mM potassium phosphate buffer twice and incubated at 30°C for 24 h in 50 mM potassium phosphate buffer that was supplemented with 0.5 mM of each substrate. One ml of culture supernatant was treated with $Hg(SCN)_2$ (0.69 g/l, 0.5 ml) and incubated for 15 min at room temperature, and then mixed with 0.5 ml of 0.25 M ferric ammonium sulfate dissolved on 4.86 N HNO_3. The concentration of releasing chloride ions was measured with a spectrophotometer (LKB4046, Pharmacia, England) at the wavelength of 453 nm as described by Bergman and Sanik (1957). The results were evaluated by comparing with blanks having no bacterial cells as described by Chae and Kim (1997).

Scanning electron microscopy

The cells from the LB agar plate were collected and prefixed with 2.5% glutaraldehyde in 100 mM potassium phosphate buffer (pH 7.2) for 2 h, and then, they were post-fixed with 1% osmium tetroxide in the same buffer for 1 h according to the methods of Ng *et al.* (1985). The fixed cells were dehydrated with a serial concentration (30 to 95%) of ethanol every 10 min, and then 100% ethanol for 20 min twice. The cells were substituted with absolute isoamyl acetate for 30 min twice, and then were air-dried. The cells were coated with gold using a sputter coater (IB-3, Giko Engineering Co., Japan) and were examined with a scanning electron microscope (S-2500C, Hitachi Ltd., Japan).

Amplification of 16S rDNA

The 16S ribosomal DNA of the isolates were analyzed by direct PCR using primers 5'-AGAGTTTGATCMTGGCTCAG-3' (27F: position 8 to 27 nt, *E. coli* 16S rDNA numbering) and 5'-TACGGYTACCTTGTTACGACTT-3' (1492R: position 1510 to 1492 nt, *E. coli* 16S rDNA numbering) which were used by Hay *et al.* (2001) for amplification of the full sequences of eubacteria.

PCR mixtures (50 μl) contained 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM concentration of each deoxynucleotide triphosphate, 50 pM of each primer, and 2.5 U of *Taq* DNA polymerase (Posco Chem, Korea). A small amount of culture cells was used as a template source for direct PCR without DNA isolation (Fode-Vaughan *et al.*, 2001). PCR amplification was performed using a Programmable Thermal Controller (MJ

Research, Inc., USA) programmed as follows: 10 min of pre-denaturation at 95°C, followed by 30 cycles (95°C for 45 sec, 55°C for 45 sec, 72°C for 1 min 30 sec), and a final extension at 72°C for 10 min.

Cloning and sequencing analysis of 16S rDNA

The amplified 1.5 kb PCR products were ligated with pGEM-T vector (Promega, USA) and were transformed into *E. coli* XL1-blue. The plasmids for sequencing were isolated with Wizard DNA purification system (Promega, USA). The DNA sequences obtained using 27F and 1492R were determined from the purified plasmid with T7, SP6 PCR primer by Labeled Primer Sequencing Methods. The 16S rDNA sequences of isolates were analyzed using the Ribosomal Database Project Sequence Match and Similarity Matrix programs (Hitachi version 7.0) in order to identify the closely matching species.

Biochemical characteristics

The biochemical characteristics were examined with API 20 NE (bioMérieux sa, France). Catalase activity of the bacterial isolates was tested with 3% H_2O_2, and oxidase activity was tested with 1% tetramethyl-p-phenylendiamine 2HCl. The resistance to antibiotics was examined using disc-diffusion methods on the agar medium as described by Manoharan *et al.* (2003).

Results and Discussion

Isolation of chloroaliphatics-degrading bacteria

From the wastewater of the Ulsan and Choengju industrial complexes, JS-79, UE-2, JS-3 and 7 other bacterial strains were isolated. The strain CA-11 was isolated from a rice paddy soil. The dechlorination of each substrate supplemented in the MM2 minimal medium for isolation is shown in Table 1. The isolated strain UE-2, UE-11 and UE-15 could degrade both 1,2-DCA and 2-MCA. On the other hand, CA-11, JS-79 and UP-18 isolated with 2-CPA as the sole carbon source could not degrade 1,2-DCA at all. However, CA-11 degraded 2,3-DCPA well, which was hardly degraded by other isolates.

When the bacterial isolates were grown on a LB agar plate at 30°C for 24 h, they could form large colonies, but UE-2 and UE-15 took 3 days to form a colony of the same size. The isolates UE-2 and UE-15 exhibited a deep yellow color and were covered with copious amounts of slime, illustrating the characteristics of *Xanthobacter*. The yellow color is known to be the zeaxanthin dirhamnoside pigment in *Xanthobacter* which produces slime consisting of glucose, galactose, mannose, and uronic acid (Wiegel and Schlegel, 1984). The capabilities of the isolates JS-66 and JS-79 being able to grow very fast and express florescent yellow substances on the LB agar media are characteristic of *Pseudomonas* group.

Identification and homology on the basis of 16S rDNA sequence

The bacterial isolates from wastewater and soil were identified on the basis of 16S rDNA sequences. The results are shown in Table 2. Seven different kinds of species were identified from eleven isolates. The isolates UE-1, UE-2, UE-11, UE-15, which can degrade 1,2-DCA, 2-MCA, and 2-CPA, were identified to belong to *Ancyclobacter sp.* and *Xanthobacter flavus*, respectively, and the isolates JS-66 and JS-79, JS-3, and JS-64 capable of degrading 2-MCA and 2-CPA, were identified to belong to *Pseudomonas, Burkholderiales,* and *Comamonas* species, respectively. Among them, UE-2 and UE-15

Table 1. Hydrolytic dechlorination of several chloroaliphatic compounds by the bacterial isolates

Isolate	Substrate for isolation	Hydrolytic dechlorination				Source
		1,2-DCA	2-MCA	2-CPA	2,3-DCPA	
JS-3	2-chloropropionic acid	-	++	++	-	Cheongju wastewater
JS-64	2-chloropropionic acid	-	++	+	-	Ulsan wastewater
JS-66	2-chloropropionic acid	-	++	+	-	Ulsan wastewater
JS-79	2-chloropropionic acid	-	++	+	-	Ulsan wastewater
UE-1	1,2-dichloroethane	++	++	+	-	Ulsan wastewater
UE-2	1,2-dichloroethane	+++	++	+	-	Ulsan wastewater
UE-11	1,2-dichloroethane	++	+	+	-	Ulsan wastewater
UE-15	1,2-dichloroethane	+++	+++	+++	-	Ulsan wastewater
CA-11	2-monochloroacetic acid	-	++	+	++	Rice paddy soil
UP-13	2,3-dichloropropionic acid	-	+	++	+	Ulsan wastewater
UP-18	2,3-dichloropropionic acid	-	+	++	+	Ulsan wastewater
Pseudomonas sp. YL	Reference strain	-	+++	+++	+	Hasan *et al.* (1994)
Xanthobacter autotrophicus GJ10	Reference strain	+++	+++	+++	-	Janssen *et al.* (1989)

1,2-DCA, 1,2-dichloroethane; 2-MCA, 2-monochloroacetic acid; 2-CPA, 2-chloropropionic acid; 2,3-DCPA, 2,3-dichloropropionic acid.
+++, good; ++, moderate; +, poor; -, none

Table 2. Identification of the bacterial isolates on the basis of 16S rDNA sequences

Isolate	Identified species	Identity %
JS-3	*Burkholderiales*, CDC Group IVc-2 str. JHH 1448	98.6
JS-64	*Comamonas testosteroni*	99.8
JS-66	*Pseudomonas plecoglossicida*	98.5
*JS-79	*Pseudomonas plecoglossicida*	99.1
UE-1	*Ancyclobacter sp. DSM 1107*	99.4
*UE-2	*Xanthobacter flavus*	99.8
UE-11	*Xanthobacter flavus*	99.9
*UE-15	*Xanthobacter flavus*	99.7
*CA-11	*Ralstonia eutropha*	99.1
UP-13	*Bosea vestrisii*	99.5
*UP-18	Alpha proteobacterium 63286	99.5

*Bacterial isolates were further examined for their morphological and biochemical characteristics.

were identified as *X. flavus* and showed closer genetic distance to each other. On the other hand, the isolates, which are able to degrade 2,3-DCPA as well as 2-MCA and 2-CPA, were identified to be *Rlastonia eutropha* (CA-11), *Bosea vestrisii* (UP-13), and Alpha proteobacteria (UP-18) species. The genetic relationships of the 16S rDNAs from the isolates UE-2, UE-15, JS-79, CA-11, and UP-13 with other species are shown in the dandrograms of Fig. 2.

In resent studies, *Pseudomonas*, *Comamonas*, and *Burkholderia* species have been isolated as soil microorganisms which can degrade a variety of chloroaliphatic compounds as well as many aromatic compounds (Schneider *et al.*, 1991; Tsang and Sam., 1999; Chae *et al.*, 2000). Many *Xanthobacter* strains were known as a 1,2-DCA degrader (Janssen *et al.*, 1985). *Ralstonia* has been reported to degrade various kinds of hydrocarbons, such as PAHs, and plays an important role in degrading various environmental pollutants (Mer-

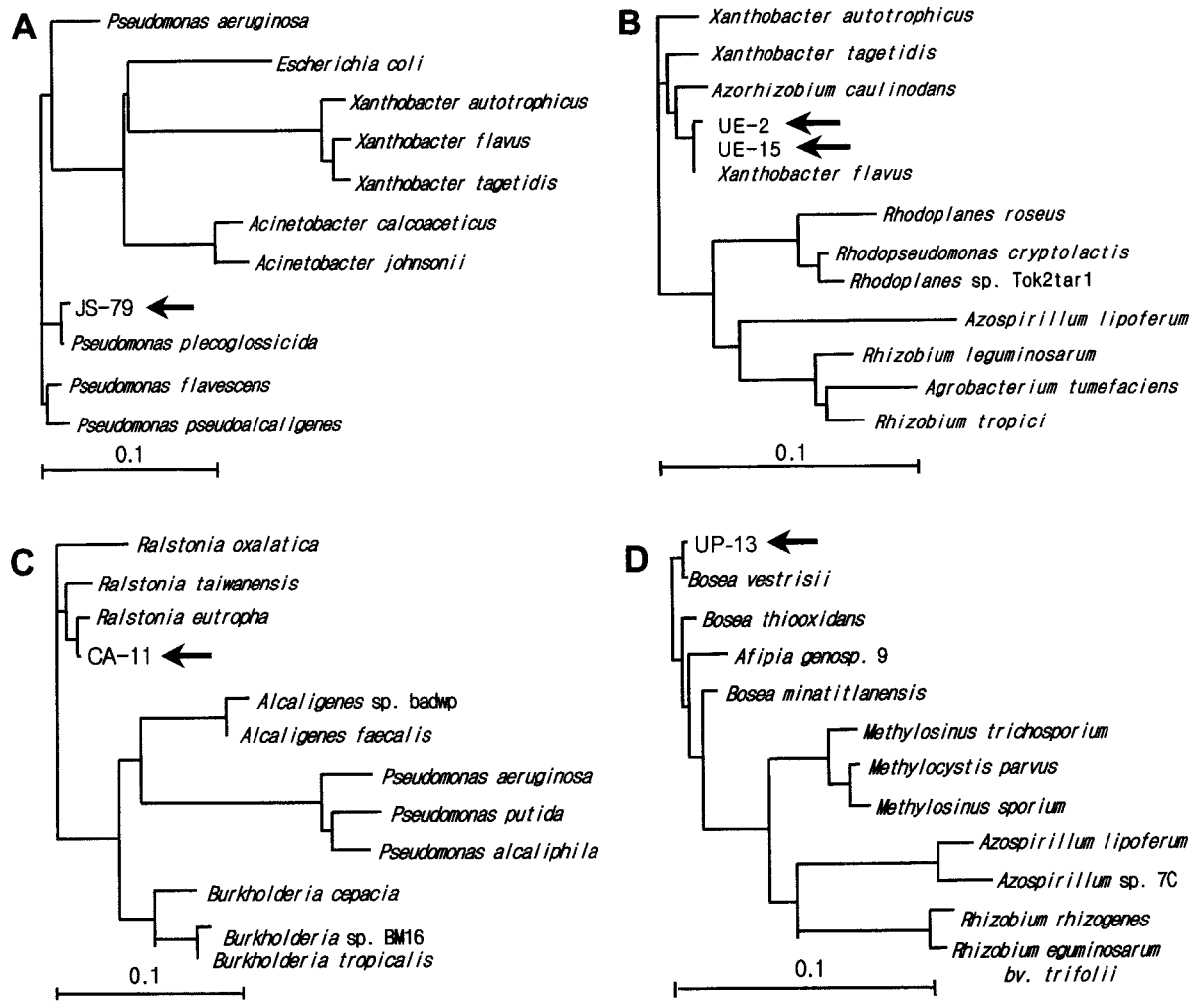

Fig. 2. Phylogenetic trees of several isolates on the basis of 16S rDNA sequences. (A), JS-79 (*Pseudomonas plecoglossicida*); (B), UE-2 (*Xanthobacter flavus*) and UE-15 (*Xanthobacter flavus*); (C), CA-11 (*Ralstonia eutropha*); (D), UP-13 (*Bosea vestrisii*). The bar indicates a 10% difference.

geay *et al.*, 2003). The results obtained in this study indicate that a variety species of bacteria with the ability to degrade various chloroaliphatic compounds as energy and carbon sources exist in our contaminated environments.

Cellular morphology and biochemical characteristics of the isolates

The cellular morphologies of the representative four isolates that were examined with an electron scanning microscope are shown in Fig. 3. The cells of EU-2 (*X. flavus*) are

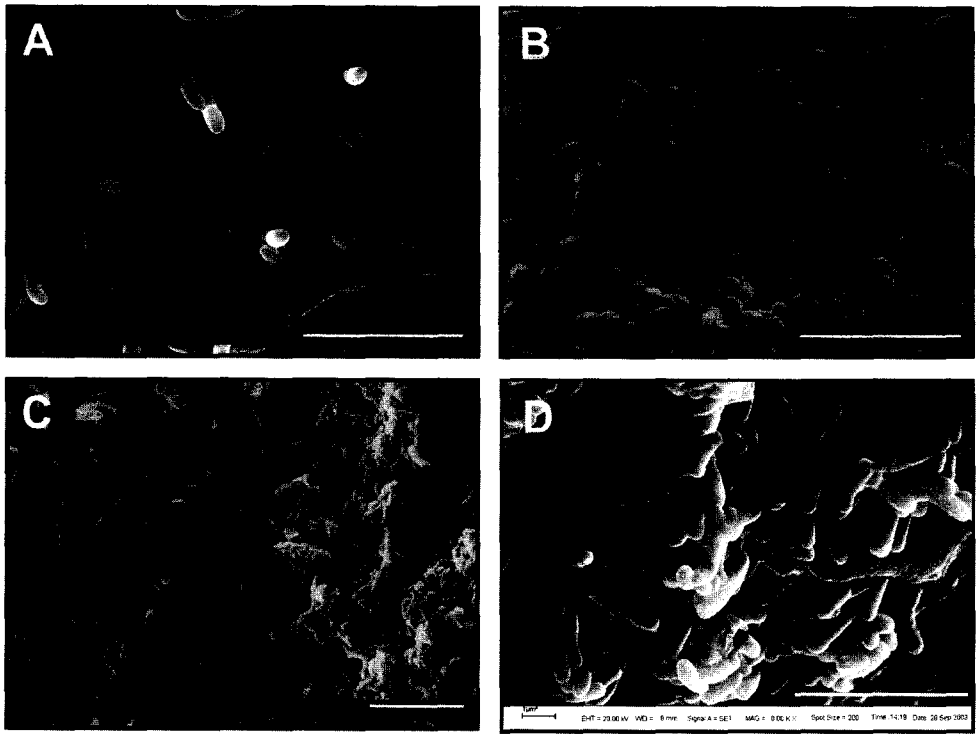

Fig. 3. Scanning electron micrographs of several bacterial isolates. (A), JS-79 (*Pseudomonas plecoglossicida*); (B), CA-11 (*Ralstonia eutropha*); (C), UE-2 (*Xanthobacter flavus*); (D), UP-18 (Alpha proteobacterium). The white bars indicate 5 μm.

Table 3. Morphological and biochemical characteristics of the bacterial isolates

Parameters	Isolates				
	JS-79	UE-2	UE-15	CA-11	UP-18
Identified species	*Pseudomonas plecoglossicida*	*Xanthobacter flavus*	*Xanthobacter flavus*	*Ralstonia eutropha*	α-proteobact
Cell shape	rod	rod	rod	coccobacillus	rod
Size (μm): Diameter	0.5±0.05	0.6±0.05	0.6±0.03	0.7±0.03	0.5±0.06
Length	2.4±0.4	2.2±0.4	2.3±0.5	1.0±0.1	2.4±0.37
Gram-staining	-	-	-	-	-
Colony	fluorescence pigment	yellow color slime formation	yellow color slime formation	bright pink color	metallic gloss
Oxygen requirement	aerobic	aerobic	aerobic	aerobic	aerobic
Oxidase	-	+	+	+	+
Catalase	+	+	+	-	+
Reduction of nitrates to nitrites	-	+	+	+	-
Tryptophane (indole production)	-	-	-	-	-
Arginine hydrolysis	+	+	+	-	-
Urea	+	+	-	+	+
Esculin hydrolysis	-	-	-	-	-
Gelatine hydrolysis	+	-	-	-	-
β-Galactosidase	-	-	-	-	-

Table 3. Morphological and biochemical characteristics of the bacterial isolates

Parameters	Isolates				
	JS-79	UE-2	UE-15	CA-11	UP-18
Identified species	*Pseudomonas plecoglossicida*	*Xanthobacter flavus*	*Xanthobacter flavus*	*Ralstonia eutropha*	α-proteobact
Assimilation					
Glucose	+	-	-	-	-
Arabinose	-	-	-	±	-
Mannose	±	-	-	-	-
Manitol	-	-	-	-	-
N-acetyl-glucosamine	-	-	-	-	-
Maltose	-	-	-	-	-
Gluconate	+	+	-	+	+
Caprate	+	-	-	+	-
Adipate	-	-	-	+	+
Malate	+	+	+	+	+
Citrate	+	+	+	-	-
Phenyl-acetate	-	-	-	-	-
Resistance to					
Ampicillin (50 μg/ml)	-	+	+	-	+
Tetracyclin (50 μg/ml)	-	-	-	-	+
Streptomycin (50 μg/ml)	+	-	-	+	++
Kanamycin (50 μg/ml)	-	+	-	+	+
Dechlorination at 30°C and pH 7.5					
2-MCA	+	+	+	+	+
1,2-DCA	-	+	+	-	-
2-CPA	+	+	+	+	+
2,3-DCPA	-	-	-	+	+

++, more positive; +, positive; -, negative; ±, weak reaction; 2-MCA, 2-monochloroacetic acid; 1,2-DCE, 1,2-dichloroethane; 2-CPA, 2-chloropropionic acid; 2,3-DCPA, 2,3-dichloropropionic acid

characteristically covered with a slime layer that could be removed by treatment with an acid or an alkaline buffer as indicated by Wiegel and Schlegel, (1984).

Five isolated strains UE-2, UE-15, CA-11, JS-79 and UP-18 identified to be *X. flavus, X. flavus, R. eutropha, Pseudomonas plecoglossicida*, and Alpha proteobacterium, respectively, were examined for their biochemical characteristics. The results are shown in Table 3.

All of the isolated bacteria grew well at 30°C and pH 7.5 under aerobic conditions, and produced chloride ions from haloaliphatic hydrocarbons by hydrolytic dechlorination. Other biochemical characteristics vary with different species. For example, the bacteria have different ranges of resistance to antibiotics, such as ampicillin, tetracycline, streptomycin, and kanamycin.

In particular, the isolates UE-2 and UE-15, identified as *X. flavus,* produced a yellow pigment in their colonies and a slime layer on the cell surfaces. Such biochemical and morphological characteristics are identical to those of *Xanthobacter* (Wiegel and Schlegel, 1984). Both of them are very sensitive to the same concentration of antibiotics than any other bacteria. The *X. flavus* strains, UE-2 and UE-15, could not use glucose, which is unlike the general characteristics of the genus *Xanthobacter,* as seen in Table 3. However, they showed different reactions in urease production, assimilation of gluconate, and resistance to kanamycin.

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