IDENTIFICATION OF SOME PREDOMINANT BACTERIA ISOLATED FROM JetA1 FUEL IN VIETNAM BY SEQUENCE ANALYSIS OF 16S rRNA GENE

Pham Thi Hang, Lai Thuy Hien, Dang Phuong Nga[1],

Daisuke Inoue, Kazunari Sei, Michihiko Ike[2]

[1] *Department of Petroleum Microbiology, Institute for Biotechnology, Hanoi, Vietnam*

[2] *Division of Sustainable Energy and Environmental Engineering, Osaka University, Japan*

Abstract

The growth of microorganisms in aviation fuel has caused fouling, malfunction and corrosion in storage tanks and has been reported since 1960. In Vietnamese tropical climate with high humidity, the contamination of microorganisms in aircraft fuel has become a serious problem. Since 1988, we have published some papers on microbial contamination in TC1 and JetA1 fuel. In this study we identified some predominant bacteria isolated from JetA1 fuel taken from aircraft wing tanks using 16S rRNA gene sequence analysis. Gram negative bacteria predominant in JetA1 fuel were members of the genera *Pseudomonas, Acinetobacter* and *Sphingomonas.* Gram positive bacteria included genera *Bacillus, Brevibacillus, Staphylococcus, Dietzia* and *Brachybacterium.* Their morphological, physiological and biochemical properties were also studied. Among them, *Pseudomonas* sp. 302-1, *Staphylococcus* sp. 302-4, *Brevibacillus borstelensis* 345-1 and *Sphingomonas* sp. 343-2 were capable of utilizing JetA1 as their carbon source. Particularly, *Pseudomonas* sp. 302-1 showed intensive growth on JetA1 and degraded up to 88% of the fuel after 3 days.

1. Introduction

The contamination of microorganisms in aviation fuel has been aware of since 1960 that caused filter plugging and corrosion of the fuel tanks, and the resultant airplane accidence may be considered as a serious consequence. We have described the microbial effects on the aviation fuel quality and characterized some predominant microorganisms in JetA1 fuel (Hien *et al.*, 1988, 1998; Hang *et al.*, 2000; Nga *et al.*, 2001). The kit for testing microbial contamination in fuel has been developed (Gaylarde *et al.*, 1999; Brian Bell, 2005). Some of them only gave a quantitative result and others helped identifying only specific microorganisms such as fuel-utilizing bacteria or only certain fungi. In recent years, we have focused on studying the diversity of contaminant microorganisms in JetA1 fuel by molecular methods in order to develop a suitable kit for a quick detection and characterization of microbial contamination in fuel. In this study, some predominant bacteria in JetA1 fuel were identified by 16S rRNA gene analysis as well as characterized by the morphological, physiological and biochemical properties.

2. Materials and methods

2.1. Materials

JetA1 fuels were collected from aircraft wing tanks following the standard sampling protocol for microbial contamination test.

2.2. Methods

2.2.1. Bacterial cultivation

Bacteria in JetA1 fuel samples were isolated on MPA medium. Isolated bacteria were routinely

grown on a mineral medium supplemented with 5% of JetA1 as sole carbon source. All bacterial cultivation was carried out at 30°C.

2.2.2. Morphological and physiological properties

Microscopic studies including colony and cell morphology, motility, measurement of cell dimension and preparation of cells for transmission electron microscopy were performed as described previously (Nga *et al*, 2001).

2.2.3. Determination of biochemical characteristics

Biochemical characterization of isolated strains was performed using API 20NE, and 50CH kit (BioMe'rieux) according to the manufacturer's instruction.

2.2.4. 16S rRNA sequence analysis

Genomic DNA was extracted from isolates according to the protocol of Sambrook and Russell (Sambrook and Russell, 2001). Partial 16S rRNA genes were amplified by PCR with universal primers (Amann *et al.*, 1995). The determination of the amplified 16S rRNA gene sequence was entrusted to TaKaRa Bio. The 16S rRNA gene sequences were compared with reference sequences using BLAST similarity searches (Altschul *et al.*, 1990).

3. Results

From 14 JetA1 fuel samples, 35 bacterial strains were isolated. Among them, 8 strains with 3 Gram negative and 5 Gram positive strains were predominant and thus selected for detailed characterization based on morphological, physiological, biochemical properties and identification by 16S rRNA gene analyses.

3.1. Characterization of Gram negative bacteria

3.1.1. Strain 302-1

Biochemical characteristics using API 20NE kit revealed that strain 302-1 had 99.9% homology to *Burkholderia cepacia* (formerly *Pseudomonas cepacia*) (Table 1). Morphological characteristics of cell and colony were very typical for genus *Pseudomonas*. Colonies were green-yellow, flat circular and entire margin. Cells had short rod shape with single polar flagellum.

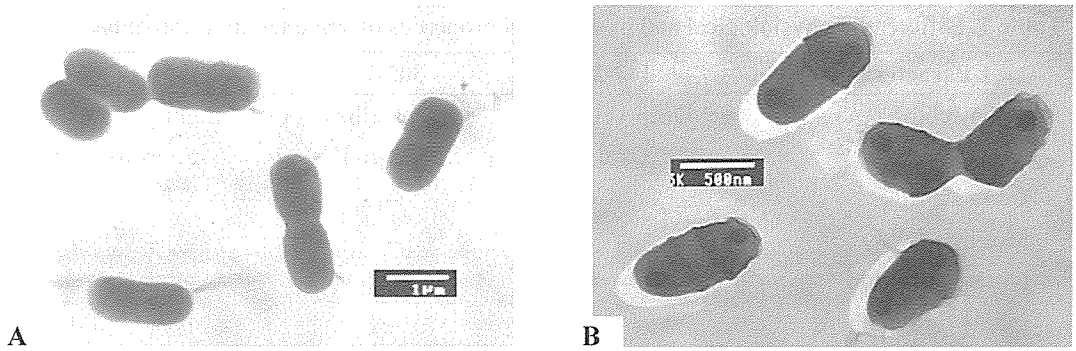
3.1.2. Strain 309-1a

Strain 309-1a showed >99% of identity value of 16S rRNA gene sequence against members of the genus *Acinetobacter* such as *Acinetobacter* sp. SS-2 (Accession No. AM396494), *A. johnsonii* F26 (EF204266), *A johnsonii* CAI-6 (DQ257426), *Acinetobacter* sp. DUT_AHX

Table 1. Differential physiological and biochemical properties of some Gram negative bacteria.

Characteristic	343-2	302-1	309-1a
Colony color	Deep-yellow	Green yellow	Light-yellow
Cell	Oval	Short-rod	Short-rod
Gram stain	−	−	−
Motility	+	+	−
Nitrate reduction	−	+	+
Indole formation	−	−	−
Glucose acidification	+w	−	−
Arginine dihydrolase	−	+	+
Urease	+w	+	+
Hydrolysis of			
Gelatine	+	+	−
β-galactosidase	−	+	−
Assimilation of			
Glucose	+	+	−
Arabinose	−	+	−
Mannose	−	+	−
Manitol	−	+	−
N-acetyl-glucosamine	−	+	−
Maltose	+	+	−
Gluconate	+	+	−
Caprate	−	+	−
Adipate	+	+	+
Malate	+	+	+w
Citrate	+	+	+w
Phenyl acetate	+	+	−
Cytochrome oxidase	−	+	−
Catalase	+	+	+

(DQ409078) and *Acinetobacter* sp. WAB1900 (AM184241). The strain also showed some morphological properties similar to *Acinetobacter* (Holt *et al.*, 1994). Colonies were light yellow, circular, convex, glistening and entire margin on complex agar medium. Cells were oval to short rod shape and not motile (Fig. 1A). Catalase, nitrate reduction, arginine dihydrolase, urease and assimilation of adipate, malate, citrate were positive but other reactions in the API 20NE kit were negative (Table 1). From the results, strain 309-1a would be a member of the genus *Acinetobacter* and possibly the species *A. johnsonii.*



A B

Fig. 1. Transmission electron micrographs of *Acinetobacter johnsonii* 309-1a (A) and *Dietzia cinnamea* 343-4 (B).

3.1.3. Strain 343-2

16S rRNA sequence of strain 343-2 showed 97-98% identity to members of *Sphingomonas* such as *Sphingomonas* sp. SKJH-30 (AY749436), *Sphingomonas* sp. S15-S1 (AM234165), *S. sanguinis* (D84529), *S. paucimobilis* ATCC 29837 (U37337) and *S. pseudosanguinis* G1-2^T (AM412238). The strain also had some morphological similarity to *S paucimobilis* and *S. sanguinis*. Colonies were deep yellow, circular and convex. Cells had oval shape and motile (Li *et al.,* 2004). Furthermore, comparing the biochemical characteristics of strain 343-2 to *S. paucimobilis* ATCC 29837^T, *S. sanguinis* IFO 13937^T and *S. pseudosanguinis* G1-2^T showed a higher similarity to *S. paucimobilis* ATCC 29837^T: catalase, gelatin hydrolysis, D-gluconate, adipate, sodium citrate assimilation were positive and oxidase, nitrate reduction, glucose acidification, caprate assimilation were negative (Table 1) (Li *et al.,* 2004; Kampfer *et al.,* 2007). Only the exceptions were N-acetyl D-glucosamine and D-mannose which were negative for the strain 343-2 but positive for strain ATCC 29837^T. These two characteristics are known to be various among the *Sphingomonas paucimobilis* species (API 20NE Analytical Profile Index, 1992).Therefore, strain 343-2 is likely a member of the genus *Sphingomonas*.

3.2. Characterization of Gram positive bacteria

3.2.1. Strain 302-4

16S rRNA gene sequence of strain 302-4 showed >99% homology to *Staphylococcus* sp. SA6 (AY864655), *S. epidermidis* HZGUAP001 (DQ268865), *S. epidermidis* (L37605), *S. epidermidis* ATCC 14990^T (D83363) and *Staphylococcaceae* bacterium KVD-unk-60 (DQ490408). Colony and cell morphology of strain 302-4 were also similar to those of *S. epidermidis* (Wiese and Han, 2000): Colonies were small, entire circular and white pigmented, and cells were spherical that occurred singly, in pairs, in short chains and irregular clusters. Thus, strain 302-4 is likely a member of genus *Staphylococcus*.

3.2.2. Strain 343-4

The sequence of 16S rRNA gene from strain 343-4 showed homology to members of genus *Dietzia*, although the highest identity values were nearly 97% against *Dietzia* sp. CIP104289 (Y08312), *D. maris* SSCS4 (AB211032), *D. maris* SSCS17 (AB211025), *D. cinnamea* IMMIB RIV-399^T (AJ920289) and *Dietzia* sp. E9_2 (AF481211). Cell and colony morphological characteristics of the strain were also similar to those of *D. maris.* and *D. cinnamea* (Rainey *et al*, 1995; Yassin *et al*, 2006): The cells were oval or short rod, none motile (Fig. 1B), and colonies were pink-yellow, circular, raised, glistening and entire margin on nutrient agar. The strain also showed similar

biochemical characteristics to *D. maris* DSM 43672^T and *D. cinnamea* IMMIB RIV-399^T as follows. All strains utilized D-glucose but did not cellobiose, L-arabinose, aesculin, D-galactose, inositol, lactose, melezitol, D-sorbitol, raffinose, rhamnose, sucrose or xylose. Strain 343-4 exhibited similarity to *D. cinnamea* IMMIB RIV-399^T but not to *D. maris* DSM 43672^T in four tests: acidification of erythritol, gluconate, mannitol and maltose (Table 2) (Rainey *et al.*, 1995; Yassin *et al.*, 2006). Analyses of 16S rRNA gene sequence and morphological and biochemical characteristic suggested that strain 343-4 may belong to the species *D. cinnamea* although more detailed phylogenetical analysis will be required for the confirmation.

3.2.3. Strain 345-1

Strain 345-1 showed >99% identity of 16S rRNA gene sequence to different *Brevibacillus borstelensis* strains. Morphological characteristic of the cells and colonies were typical for the species *B. borstelensis* proposed by Shida *et al.* (1996). Cells were irregular oval and motile. Colonies were butyrous orange, flat and glistening with irregular margin. The 16S rRNA sequence and morphological analyses suggested that strain 345-1 may be *Brevibacillus borstelensis.*

3.2.4. Strain 345-2

16S rRNA gene sequence of strain 345-2 showed >99% similarity to the species *Bacillus subtilis*. In addition, the strain exhibited typical morphological characteristics of *B. subtilis* (Holt *et al.*, 1994). Cells had rod shape occurred in chains and formed spores. Colonies were flat with yellow-white color and irregular margin. Hence, strain 345-2 presumably belongs to the species *B. subtilis.*

3.2.5. Strain 502-4

Strain 502-4 had >99% similarity of 16S rRNA gene sequence to different members of the genus *Brachybacterium*, particularly the species *B. paraconglomeratum*. Morphological characteristics of strain 502-4 seemed to be similar to *B. paraconglomeratum* (Takeuchi *et al*, 1995): Cells were rod with round ends, non-motile and non-sporing. Furthermore, biochemical characteristics of the strain showed a high similarity to *B. paraconglomeratum* LMG 19861^T. Catalase activity, acid production from L-arabinose, D-fructose, D-galactose, D-glucose, maltose, lactose, D-mannose, melezitose, sucrose, adonitol, and inositol were positive. Oxidase activity, acid production from D-arabinose, raffinose, D-ribose, and xylitol were negative. However, there were contradict characteristics in acid production from salicin, trehalose, D-xylose, mannitol, sorbitol (Table 2) (Takeuchi *et al*, 1995). From the results, strain 502-4 would be a member of the genus *Brachybacterium*.

Table 2. Differential physiological and biochemical properties of strains 502-4 and 343-4

Characteristics	502-4	343-4	Characteristics	502-4	343-4
Colony colour	White	Pink	Amygdalin	–	+
Cell	Round end-rod	Oval	Arbutin	–	+w
Gram stain	+	+	Esculin	+w	–
Motility	–	–	Salicin	–	–
Catalase	+w	+	D-cellobiose	–	–
Acid production from			D-maltose	+	+
Glycerol	+	+w	D-lactose	–	+w
Erythritol	–	–	D-melibiose	–	–
D-arabinose	–	–	D-saccharose	+	–
L-arabinose	+	–	D-trehalose	+w	+
D-ribose	–	–	Inulin	–	+
D-xylose	+	–	D-melezitose	+	–
L-xylose	–	–	D-raffinose	–	–
D-Adonitol	+	+	Amidon	–	–
Methyl-β D-Xylopyranoside	–	+w	Glycogen	+w	–
D-galactose	+	–	Xylitol	–	–
D-glucose	+	+	Gentiobiose	–	–
D-fructose	+	+	D-turanose	+	–
D-manose	+	–	D-lyxose	–	–
L-sorbose	+w	–	D-tagatose	–	+w
L-rhamnose	–	–	D-fucose	–	–
Dulcitol	–	–	L-fucose	+	–
Inositol	+w	+w	D-arabitol	+	–
D-mannitol	+	–	L-arabitol	–	–
D-sorbitol	+	–	Potassium Gluconate	–	–
Methyl-β D-Manopyranoside	–	–	Potassium 2-Ketogluconate	–	–
Methyl-β D-Glucopyranoside	+	–	Potassium 5-Ketogluconate	+	+
N-acetyl Glucosamine	+	–			

3.3. JetA1 utilizing ability

All selected isolates were characterized for their JetA1 utilization ability. However, only strain 302-1 showed intensive growth on JetA1 fuel as sole carbon and energy source, and exhibited an increase of 5×10^5 fold of cells after 3 days when incubated with JetA1. Gas chromatography analyses also revealed that the total saturated hydrocarbons as well as different hydrocarbon chains from C9-C18 decreased significantly (Table 3). Three other strains, *Staphylococcus* sp. 302-4, *Brevibacillus borstelensis* 345-1 and *Sphingomonas* sp. 343-2, also showed moderate growth on JetA1 fuel.

4. Discussion

Results of above analyses showed that predominant Gram negative bacteria contaminated in JetA1 fuel were *Pseudomonas* sp., *Acinetobacter johnsonii* and *Sphingomonas* sp. Contaminated Gram positive bacteria included *Staphylococcus* sp., *Dietzia cinnamea, Bacillus subtilis, Brachybacterium* sp. and *Brevibacillus borstelensis*. Some of these genera have been previously detected in fuels (Edmonds and Cooney, 1967; Rauch *et al.*, 2006). However, this is the first report on the isolation of members of the genera *Acinetobacter*, *Brachybacterium* and *Brevibacillus* from fuels.

JetA1 fuel utilization tests in this study revealed that among the predominant bacteria only strain 302-1 could utilize the fuel as sole carbon and energy source. This is consistent with our previous studis which clarified that among the contaminated bacteria in JetA1 fuel *Pseudomonas* spp. were JetA1-utilizing bacteria and *Bacillus* spp. utilized not JetA1 fuel but the intermediates which produced by the biodegradation of JetA1 fuel (Hang *et al.*, 2000; Nga and Hien, 2001). Edmonds and Cooney (1967), who detected some members of the genera *Pseudomonas, Staphylococcus* and *Brevibacterium* in JP-4 fuel, also showed that *Pseudomonas* spp. could utilize JP-4 as sole carbon source but *Bacillus* spp. represented over one third of the organisms isolated did not. Rauch *et al.* (2006), who detected viable bacteria of *Bacillus* sp., *Sphingomonas* sp., *Staphylococcus* sp. and *Dietzia* sp. in USAF aircraft fuel using genetic and GC-FAME techniques, suggested that *Bacillus* spp. might play a role in liberating hydrocarbons from the fuel phase into the aqueous phase for metabolisms by their ability of producing biosurfactant. These facts suggest that predominant bacteria isolated in this study other than strain 302-1 involves in (1) the liberation of hydrocarbons in JetA1 fuel to aqueous phase before their degradation by strain 302-1 and other JetA1-utilizing bacteria that could not isolated in this study and (2) the degradation of intermediates produced during the biodegradation of JetA1 fuel.

Table 3. Gas chromatography analysis of saturated hydrocarbons of JetA1 after 72 hours incubated with strain 302-1.

Sample	Saturated hydrocarbon (ppm)										
	Total	C9	C10	C11	C12	C13	C14	C15	C16	C17	C18
Negative control	16052	134	2608	3878	4024	2808	1652	632	206	92	18
Strain 302-1	3546	5	362	780	970	766	482	137	36	7	0

References

1. API 20NE Analytical Profile Index (1992) 5th Edition.
2. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., Lipman, D. J. (1990) Basic local alignment search tool. J. Mol. Biol. 215(3): 403-410.
3. Amann, R. I., Ludwig, W., Schleifer, K. H. (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59(1): 143-169.
4. Brian Bell (2005) Asia Pacific Aviation News 28 November, 2005. London Press.
5. Edmonds, P., Cooney, J. (1967) Identification of microorganisms isolated from jet fuel systems. Appl. Microbiol. 15(2): 441-416.
6. Gaylarde, C. C., Bento, F. M., Kelly, J. (1999). Microbial contamination of stored hydrocarbon fuels and its control. Rev. Microbiol. 30(1): 01-10.
7. Hang, P. T., Hien, L. T., Quyen, N. D. (2000) Taxonomic characteristics and hydrocarbon - degradation ability of some Gram negative bacteria strains isolated from the Jet A1 fuel. J. Biology. 22(4): 31-37.
8. Hien, L. T., Ha, D. C., Bang, L. K., Hai, N. T. (1988) Microbial contamination in TC1 fuel, in Vietnam. J. Biol. 10(1): 5-9.
9. Hien, L. T., Phuong, D. T., Anh, V. P., Nga, D. P., Thuy, P. T., Hai, H., Hang, P. T. (1998) Microorganisms in Jet A1 fuel and alternative eliminating by biocides. Annual Report of IBT, NCST. Publishing House Science and Technique Hanoi 2000.
10. Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T., Williams, S. T. (1994) Bergey's manual of determinative bacteriology. Library of congress cataloging in publication data. USA.
11. Kampfer P, Uwe M, Michael E, Thomas H, Hans JB (2007) *Sphingomonas pseudosanguinis* sp. nov., isolated from the water reservoir of an air humidifier. Int. J. Syst. Evol. Microbiol. 57(6): 1342-1345.
12. Li, Y., Kawamura, Y., Fujiwara, N., Naka, T., Liu, H., Huang, X., Kobayashi, K., Ezaki, T. (2004) *Sphingomonas yabuuchiae* sp. nov. and *Brevundimonas nasdae* sp. nov., isolated from the Rusian space laboratory Mir. Int. J. Syst. Evol. Microbiol. 54(3): 819-825.
13. Nga, D. P., Hien, L. T. (2001) Taxonomic characteristics of some acquainted positive Gram bacteria strains and their effects on the quality of Jet A1fuel. J. Biology. 23(2): 20-26.
14. Rainey, F. A., Klatte, S., Kronppenstedt, R. M., Stackebrandt, E. (1995). *Dietzia,* a new genus including *Dietza maris* comb. nov., formerly *Rhodococcus maris*. Int. J. Syst. Bacteriol. 45(1): 32-36.
15. Rauch, M. E., Graef, H. W., Rozenzhak, S. M., Jones, S. E., Bleckmann, C. A., Kruqer, R. L., Naik, R. R., Stone, M. O. (2006) Characterization of microbial contamination in United States Air Force aviation fuel tanks. J. Ind. Microbiol. Biotechnol. 33(1): 29-36.
16. Sambrook J, Russell DW (2001) Molecular Cloning. A Laboratory Manual, 3 rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
17. Shida O, Takagi, H., Kadowaki, K., Komagata, K. (1996) Proposal for two new genera, *Brevibacillus* gen. nov. and *Aneurinibacillus* gen. nov. Int. J. Syst. Bacteriol. 46(4): 939-946.
18. Takeuchi, M., Fang, C. X., Yokota, A. (1995) Taxonomic study of the genus *Branchybacterium:* proposal of *Branchybacterium conglomeratum* sp. nov., nom. rev., *Branchybaterium paraconglomeratum* sp. nov., and *Branchybacterium rhamnosum* sp. nov. Int. J. Syst. Bacteriol. 45(1): 160-168.
19. Wieser, M., Busse, H. J. (2000) Rapid identification of *Staphylococcus epidermidis*. Int. J. Syst. Evol. Microbiol. 50(3): 1087-1093.
20. Yassin, A. F., Hupfer, H., Schaal, K. P. (2006) *Dietzia cinnamea* sp. nov., a novel species isolated from a perianal swab of a patient with a bone marrow transplant. Int. J. Syst. Evol. Microbiol. 56(3): 641-645.