

## *Jannaschia cystaugens* sp. nov., an *Alexandrium* (Dinophyceae) cyst formation-promoting bacterium from Hiroshima Bay, Japan

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Heterotrophic bacteria isolated from water samples taken from Hiroshima Bay, Japan, and referred to as *Alexandrium* (Dinophyceae) cyst formation-promoting bacteria, were assigned to the *Roseobacter–Sulfitobacter–Silicibacter* group within the  $\alpha$ -*Proteobacteria* on the basis of nearly complete 16S rRNA gene sequences. Phylogenetic analyses showed that two strains, CFPB-A9<sup>T</sup> and CFPB-A5, are closely related to each other and that their closest relative was *Jannaschia helgolandensis* (95.9% sequence similarity). These strains were Gram-negative, motile, obligately aerobic rods that required sodium ions and 2–7% sea salts for growth and did not produce bacteriochlorophyll *a*. Their optimal growth temperature was 25–30 °C. The strains had Q-10 as the dominant respiratory quinone. Primary cellular fatty acid in both strains was 18:1 $\omega$ 7c. The DNA G+C contents of strains CFPB-A9<sup>T</sup> and CFPB-A5 were 59.1 and 59.2 mol%, respectively. Based on physiological, biological, chemotaxonomic and phylogenetic data, the strains are considered to represent a novel species, *Jannaschia cystaugens* sp. nov., with type strain CFPB-A9<sup>T</sup> (=LMG 22015<sup>T</sup>=NBRC 100362<sup>T</sup>).

Recently, the existence of *Alexandrium* (Dinophyceae) cyst formation-promoting bacteria (Alex-CFPB) in Hiroshima Bay, Japan, where the bloom-forming toxic species *Alexandrium tamarense* occurs annually, was reported by Adachi *et al.* (1999). They found a clear positive correlation between the abundance of Alex-CFPB and *A. tamarense* and suggested that Alex-CFPB may play a significant role in the process of encystment and bloom disintegration in the field (Adachi *et al.*, 1999). Furthermore, Adachi *et al.* (2003) reported that, among the strains of the 31 Alex-CFPB isolated, a total of 14 ribotypes, from ribotype A to ribotype N, were determined by restriction fragment length polymorphism (RFLP) analysis of their partial 16S rRNA gene. They clarified that most of the Alex-CFPB belonged to the  $\alpha$ -*Proteobacteria*, the *Roseobacter–Sulfitobacter–Silicibacter* group reported by Wagner-Döbler *et al.* (2003), and the

bacterial strains of ribotype A among them were suggested to have been dominant during the periods of bloom peak and bloom decay of *A. tamarense* (Adachi *et al.*, 2003). A large number of strains from the *Roseobacter–Sulfitobacter–Silicibacter* group have been cultivated from various marine environments over the last few years. Strains belonging to the species *Roseobacter denitrificans* and *Roseobacter litoralis* are strict aerobes that are capable of photosynthesis using bacteriochlorophyll *a* (Shiba, 1991). Aerobic heterotrophs from the group include members of the genera *Octadecabacter* (Gosink *et al.*, 1997), *Sulfitobacter* (Labrenz *et al.*, 2000; Pukall *et al.*, 1999; Sorokin, 1995), *Sagittula* (González & Moran, 1997), '*Marinosulfonomonas*' (Holmes *et al.*, 1997), *Leisingera* (Schaefer *et al.*, 2002), *Antarctobacter* (Labrenz *et al.*, 1998), *Roseovarius* (Labrenz *et al.*, 1999), *Ruegeria* (Uchino *et al.*, 1998), *Ketogulonicigenium* (Urbance *et al.*, 2001), *Roseivivax* (Suzuki *et al.*, 1999), *Silicibacter* (Petursdottir & Kristjansson, 1997), *Staleyia* (Labrenz *et al.*, 2000) and *Jannaschia* (Wagner-Döbler *et al.*, 2003). Physiological characteristics of the species within these genera are diverse, ranging from degradation of lignin (González & Moran, 1997), degradation of aromatic compounds (Buchan *et al.*, 2000), degradation of dimethyl sulfonio-propionate produced by algae and coastal vascular plants (González *et al.*, 1999; Ledyard *et al.*, 1993) to transformations of organic and inorganic sulfur compounds (Holmes

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**Abbreviation:** Alex-CFPB, *Alexandrium* cyst formation-promoting bacteria (-um).

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Jannaschia cystaugens* CFPB-A9<sup>T</sup> and CFPB-A5 are AB121782 and AB121783, respectively.

Fatty acid profiles for strains CFPB-A9<sup>T</sup> and CFPB-A5 are available as supplementary material in IJSEM Online.

*et al.*, 1997; Kiene *et al.*, 1999; Pukall *et al.*, 1999). Here, we characterize and describe an Alex-CFPB of ribotype A, strains CFPB-A9<sup>T</sup> and CFPB-A5, from Hiroshima Bay, which may be dominant among the Alex-CFPB assemblages and potentially play an important ecological role in the process of encystment of *A. tamarense* in this bay.

Two bacterial strains (CFPB-A9<sup>T</sup> and CFPB-A5) were isolated from coastal sea-water samples taken at station 11 (Stn 11, depth about 22 m), a shallow coastal site in Hiroshima Bay, western Seto Inland Sea. Bacterial sampling was conducted on 28 April and 21 May 1998 according to the method described previously (Adachi *et al.*, 1999). Strain CFPB-A9<sup>T</sup> was obtained from the diluted supernatant from a 'most probable number (MPN)-positive well' containing the sea-water sample from Hiroshima Bay taken on 28 April, as described previously (Adachi *et al.*, 2003). For an MPN assay, each sea-water sample was first filtered through a glass-fibre filter and then through a 0.8 µm Millipore or 0.2 µm Nuclepore filter. The 0.8 µm filtrate was considered as the fraction containing the bulk of the planktonic bacteria (BF) and the 0.2 µm filtrate as the 'bacteria-free' fraction (BFF). The BF filtrate was serially diluted. BFF, BF, serially diluted BF and f/2-Si medium (Adachi *et al.*, 1996) were each inoculated into 16 wells of 48-well disposable sterilized tissue-culture microplates (Iwaki CO). In parallel, axenic cultures of *Alexandrium catenella* 6ax and TNY7 in the mid-exponential phase of growth were mixed together, as described by Adachi *et al.* (1999). One millilitre of the mixed cell suspension was inoculated into each of the 48 microwells, mixed gently and incubated under the conditions described by Adachi *et al.* (1999). One month after the start of the incubation, the numbers of whole cysts formed in each well were counted under an inverted microscope (IX-FLA; Olympus). Wells in which the cyst number was more than three times higher than the mean cyst number formed in the bacteria-free wells ( $n=18$ ) were regarded as 'Alex-CFPB-positive'. One hundred microlitres of these diluted MPN-positive samples were spread onto FeTY agar plates [0.01 g iron(III) citrate l<sup>-1</sup>, 0.5 g trypticase peptone l<sup>-1</sup>, 0.05 g yeast extract l<sup>-1</sup>, 15 g agar l<sup>-1</sup>, 80 % sea water] and incubated at 20 °C for 1 month in the dark using the method described by Adachi *et al.* (2001). Strain CFPB-A5 was isolated directly from the sea-water sample taken on 21 May using FeTY agar under the conditions described previously (Adachi *et al.*, 2003). The cyst formation-promoting activities (CFPAs) of CFPB-A9<sup>T</sup> and CFPB-A5, calculated using the formula  $CFPA = \text{cyst number in wells inoculated with bacteria (bacterial wells)}/\text{cyst number in bacteria-free wells}$ , were 11.6 and 8.01, respectively (Adachi *et al.*, 2003). CFPAs of these strains were determined repeatedly using the method described previously.

Growth was typically observed for strains CFPB-A9<sup>T</sup> and CFPB-A5 after 5 days on Marine Agar 2216 (Difco) plates when incubated at 25 °C. Colonies of these two strains were circular and convex with a cream-coloured margin and a raised, dark-brown centre. Older colonies of these strains

became brown. Morphological characterization was carried out using light microscopy, including phase-contrast observations. Cells were irregular rods (sizes are given in Table 1). Transmission electron microscope investigation was carried out as described by Rheims *et al.* (1999). White inclusion bodies were sometimes present, which were clearly not gas vesicles, as judged by transmission electron microscope investigations (Fig. 1). The two strains did not form spores. A culture of the type strain *Jannaschia helgolandensis* Hel 10<sup>T</sup> was obtained from NCIMB Japan Co., Ltd (Shizuoka, Japan) and was used as a reference strain. All strains were stored at -80 °C in Marine Broth 2216 (Difco) supplemented with 20 % (v/v) glycerol.

A loopful of cell material of strains CFPB-A9<sup>T</sup> and CFPB-A5 was prepared from cultures in the late-exponential phase of growth that had been incubated in Marine Broth 2216 (Difco) for 5 days and then harvested by centrifugation at 8000 g for 10 min. For each of the strains, a suspension corresponding to McFarland standard 1 (OD<sub>550</sub> 0.25; bioMérieux) was prepared in 10 ml saline buffer (0.85 % NaCl) supplemented with 2 % (w/v) sea salts (Sigma) as described by Wagner-Döbler *et al.* (2003). One drop of this suspension was added to each of the test tubes or test plates. Temperature range for growth was tested in Marine Broth 2216 at 5, 10, 15, 20, 25, 30, 37 and 45 °C. Growth was poor at 5 °C and continued up to 30 °C, with an optimum temperature range of 25–30 °C. Halotolerance was tested in LBSS medium (10.0 g tryptone l<sup>-1</sup>, 5.0 g yeast extract l<sup>-1</sup>) with 0, 1, 2, 3, 5, 7, 10 and 13 % (w/v) sea salts (Sigma) added as described by Wagner-Döbler *et al.* (2003). In medium devoid of sea salts, no growth of either isolate was observed. Growth was observed at sea-salt concentrations of 2–7 % (w/v), with optimum growth at 3–5 %. The pH range for growth was determined in the range 6.0–10 in steps of one pH unit using Marine Broth 2216. Growth was observed at pH 7.0–9.0, with optimum growth at pH 7.0. These tests were carried out in triplicate. Capacity for anaerobic growth was tested on Marine Agar 2216 incubated in an Anaero Pack (Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan). Both strains grew strictly aerobically. The following tests were carried out according to the methods described by Gerhardt *et al.* (1994): Gram-staining, motility, cytochrome oxidase reaction, catalase reaction, starch hydrolysis, gelatin hydrolysis and nitrite production. Glucose fermentation was tested using the fermentation medium reported by Leifson (1963). The results are summarized in Table 1. Physiological reactions were tested using the substrate panels of the API 20NE and API 50CH systems (bioMérieux) according to the manufacturer's specifications, except that sea salts (Sigma) were added to the inocula. For the inocula of the API 20NE and API 50CH assimilation tests, 2.8 ml of 10 % (w/v) sea salts and 7.2 ml of AUX medium were added to the 10 ml of the cell suspension corresponding to McFarland standard 1 described above. Each strip was tightly sealed with Parafilm and incubated at 25 °C for 20 days. In the physical and biochemical tests, *J. helgolandensis* Hel 10<sup>T</sup> was used as a

**Table 1.** Differential characteristics of strains CFPB-A9<sup>T</sup> and CFPB-A5 and *J. helgolandensis*

Both species were Gram-negative and catalase-positive, and negative for glucose fermentation and hydrolysis of gelatin or starch. Neither reduced nitrate to nitrite. They did not produce indole, urease or arginine dihydrolase. All strains were positive for assimilation of glycerol, D-glucose, mannitol, sorbitol, starch, D-arabitol and gluconate. All strains were negative for assimilation of caprate, adipate, citrate, phenylacetate, erythritol, D-arabinose, L-arabinose, adonitol, methyl  $\beta$ -xyloside, L-sorbose, dulcitol, methyl  $\alpha$ -D-mannoside, methyl  $\alpha$ -D-glucoside, N-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, D-raffinose, glycogen, xylitol,  $\beta$ -gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, L-arabitol and 5-ketogluconate. +, Positive; -, negative; w, weakly positive. Data for *J. helgolandensis* were taken from Wagner-Döbler *et al.* (2003), unless indicated otherwise.

Characteristic	CFPB-A9 <sup>T</sup> and CFPB-A5	<i>J. helgolandensis</i> Hel 10 <sup>T</sup>
Cell length ( $\mu\text{m}$ )	1.5–2.0	1.9–3.2
Cell diameter ( $\mu\text{m}$ )	0.5–0.8	0.7–1.1
Motility	+	–
Temperature range for growth ( $^{\circ}\text{C}$ )	5–30	15–30
Growth in 1.0% sea salts	–	+
Growth at pH 9.0	+	–
Cytochrome oxidase	+	w
Hydrolysis of:		
Aesculin	–	w*
<p>-Nitrophenol-<math>\beta</math>-D-galactopyranoside</p>	–	+*
Utilization of:		
Malate, ribose, D-xylose, L-xylose, galactose, D-fructose, D-mannose, rhamnose, inositol, cellobiose, 2-ketogluconate	–	+
DNA G+C (mol%)	59.1, 59.2	63.0

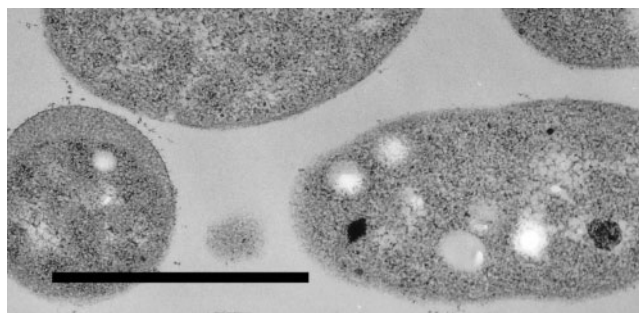
\*API 20NE data for strain Hel 10<sup>T</sup> from this study.

reference strain. Biochemical and physiological characteristics useful for identifying and differentiating the novel isolates and *J. helgolandensis* are shown in Table 1.

The absorption spectra of whole cells (Shiba, 1991) and methanol extracts (Ledyard *et al.*, 1993) of ultrasonicated cells were determined with a Hitachi F-2000 spectrophotometer. Bacteriochlorophyll *a* was not detected by *in vivo* spectroscopy or by *in vitro* spectroscopy in strains CFPB-A9<sup>T</sup> and CFPB-A5. Isolation of genomic DNA was carried out according to the method of Pitcher *et al.* (1989). The DNA base composition was determined by HPLC after enzymic digestion of DNA to deoxyribonucleosides

(Katayama-Fujimura *et al.*, 1984). A DNA-GC kit (Yamasa Shoyu, Tokyo, Japan) was used as a quantitative standard. The DNA G+C contents of the two strains, CFPB-A9<sup>T</sup> and CFPB-A5, seem to be substantially different from those of *J. helgolandensis* analysed by Wagner-Döbler *et al.* (2003) (Table 1). DNA–DNA hybridization experiments were performed in microdilution wells using a fluorometric binding method (Ezaki *et al.*, 1988). The experiments showed a high level of DNA–DNA relatedness (99.2%) between strains CFPB-A9<sup>T</sup> and CFPB-A5, and low levels of relatedness between *J. helgolandensis* and CFPB-A9<sup>T</sup> (20.5%) and between *J. helgolandensis* and CFPB-A5 (19.1%).

Quinones were extracted with chloroform/methanol (2:1, v/v) from cells of strains CFPB-A9<sup>T</sup> and CFPB-A5 grown for 5 days in Marine Broth 2216 at 25  $^{\circ}\text{C}$ . The extract was purified by the method described by Hiraishi *et al.* (1996) and analysed using a Shimadzu LC-10 HPLC system (Shimadzu, Tokyo, Japan). Analysis of the respiratory quinone composition indicated that Q-10 predominated in both strains. Fatty acid profiles of CFPB-A9<sup>T</sup> and CFPB-A5, and of *J. helgolandensis* Hel 10<sup>T</sup> as a reference strain, were quantified and identified by comparison to a commercial database, the Sherlock Microbial Identification System (MIS) by Microbial ID (MIDI) using the manufacturer's protocols. Isolate identifications were made by comparison to the MIDI TSBA (version 4.0) microbial database as described by Urbance *et al.* (2001). Cells used for the fatty acid analysis were grown on Marine Agar 2216 at



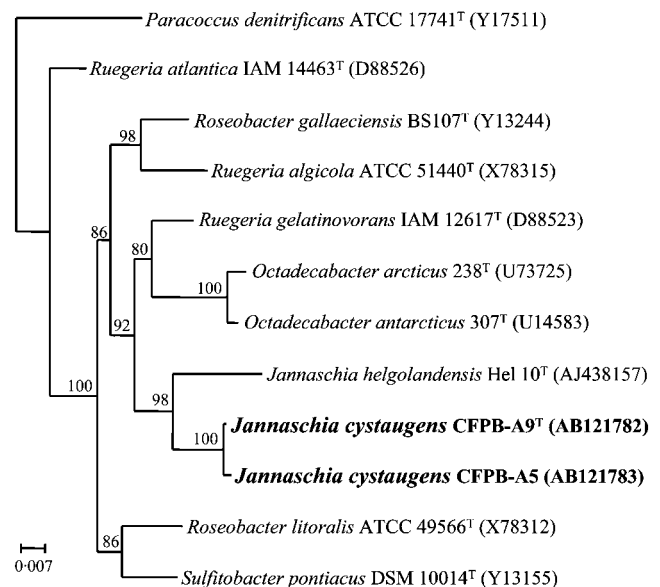
**Fig. 1.** Transmission electron micrograph of cells of strain CFPB-A9<sup>T</sup>. Bar, 1  $\mu\text{m}$ .

25 °C for 48 h. Fatty acid profiles for CFPB-A9<sup>T</sup>, CFPB-A5 and *J. helgolandensis* Hel 10<sup>T</sup> were dominated by 18:1 $\omega$ 7c (>54%). The profiles of these three strains had minor contributions from 3-OH 10:0 (3–8%), unknown 11.799 (2–7%), 18:0 (2–9%) and methyl 18:1 $\omega$ 7c (2–9%) fatty acids. The similarity in fatty acid profiles among CFPB-A9<sup>T</sup>, CFPB-A5 and *J. helgolandensis* Hel 10<sup>T</sup> supports the CFPB strains as belonging to the genus *Jannaschia*. Significant differences in major (>10% of total recovered fatty acids) or in minor (1–10%) fatty acids were not observed between CFPB-A9<sup>T</sup> and CFPB-A5. However, cyclo 19:0 $\omega$ 7c fatty acids were found in *J. helgolandensis* Hel 10<sup>T</sup> (17.7%) but not in the two CFPB strains. Fatty acid profiles for the strains are available as supplementary material in IJSEM Online. In the case of *J. helgolandensis* Hel 10<sup>T</sup>, fatty acids 12:1, 17:0 and 20:0 were not detected by the Microbial ID system, whereas they had been detected in this strain by the analysis of Wagner-Döbler *et al.* (2003) using the method reported previously by Labrenz *et al.* (1998).

Bacterial genomic DNA was extracted according to the method described above. Sequences of the near-complete 16S rRNA gene were PCR-amplified using the 27F primer and the 1492R primer according to the method of Adachi *et al.* (2001). Completed amplifications were purified and quantified according to the method described by Adachi *et al.* (1996). PCR products were deprimarized using a GeneClean III Kit according to the manufacturer's recommendations (Bio101). The purified products were sequenced directly using a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (PE Biosystems), with bacterial universal 27F primer, and primers EUB338F, EUB338R, BAC514F, BAC514R, BAC785R, BAC1059F, BAC1059R, BAC1375F, BAC1375R and 1492R described previously (Adachi *et al.*, 2001) and a newly designed primer, BAC785F (5'-GGATTAGATACGCTGGTAGTC-3'). The number in each primer name shows the corresponding position in the *Escherichia coli* 16S rRNA gene. DNA sequences were read directly to a computer using ABI PRISM 310 Genetic Analyser (PE Biosystems). Almost the entire 16S rRNA gene sequences were aligned to various bacterial 16S rRNA gene sequences obtained from the EMBL, GenBank and DDBJ databases using the software CLUSTAL W (version 1.6; EMBL). Distances were inferred from sequences using the one-parameter model (Jukes & Cantor, 1969). Branch lengths of phylogenetic trees were evaluated using the neighbour-joining method (Saitou & Nei, 1987) using CLUSTAL W and maximum-likelihood methods using DnaML, within the PHYLIP package (Felsenstein, 1995). Bootstrap analyses of 1000 replicates were carried out. Phylogenetic inferences were based on analysis of nearly complete 16S rRNA gene sequences of strains CFPB-A9<sup>T</sup> and CFPB-A5 (*E. coli* positions 49–1450). The 16S rRNA gene sequences of strains CFPB-A9<sup>T</sup> and CFPB-A5 were nearly identical (99.7% similarity). The high similarity suggests that CFPB-A9<sup>T</sup> belongs to the same species as CFPB-A5. This was supported by the

similarity in the DNA G+C contents of the two strains as well as the high level of DNA–DNA relatedness of these two strains, described above. Phylogenetic analyses revealed that the strains were members of the  $\alpha$ -Proteobacteria and were associated with the *Roseobacter–Sulfitobacter–Silicibacter* group described by Wagner-Döbler *et al.* (2003) (= *Roseobacter* group reported by González *et al.*, 2000). Their nearest phylogenetic neighbour was *J. helgolandensis* Hel 10<sup>T</sup> (95.9% sequence similarity). This similarity value (<97%), the difference in their G+C contents and the low level of DNA–DNA relatedness between the CFPB strains and *J. helgolandensis* suggest that the CFPB strains represent a species different from *J. helgolandensis*. A phylogenetic tree of 16S rRNA gene relationships, displaying the positions of strains CFPB-A9<sup>T</sup> and CFPB-A5 relative to their neighbours, is shown in Fig. 2. Phylogeny shows that the CFPB strains always grouped with *J. helgolandensis* when different ingroups were included in the phylogenetic analysis with the neighbour-joining method (Fig. 2). The topology of the tree was confirmed using maximum-likelihood analyses (data not shown). These topologies were supported by high bootstrap values (98%). These results suggest that the new strains belong to a novel species of the genus *Jannaschia*.

Several phenotypic features and fatty acid profiles allowed differentiation between the new strains and *J. helgolandensis*. In addition, the differences in G+C contents, the low level of DNA–DNA relatedness and phylogenetic divergence



**Fig. 2.** Phylogeny of the type species in the *Roseobacter–Sulfitobacter–Silicibacter* lineage in the  $\alpha$ -Proteobacteria and representative members outside the group. The tree was generated by using the neighbour-joining method. Numbers at nodes indicate percentage bootstrap values above 50 (1000 replicates). Positions 49–1450 (*E. coli* numbering) were considered. Bar, 0.007 nt substitution per site.

between them justify placing the newly described strains in a novel species of the genus *Jannaschia*, for which we propose the name *Jannaschia cystaugens* sp. nov.

### Description of *Jannaschia cystaugens* sp. nov.

*Jannaschia cystaugens* [cyst.au'gens. Gr. n. *cystis* cyst; L. part. adj. *augens* promoting; N.L. part. adj. *cystaugens* promoting cyst (formation)].

Cells are Gram-negative, non-spore-forming, strictly aerobic and heterotrophic. Motile, irregular rods, 1.5–2.0 µm long and 0.5–0.8 µm wide. White inclusion bodies are sometimes observed. Oxidase-positive. Catalase-positive. Genomic DNA G+C content is 59.1–59.2 mol% (as determined by HPLC). Growth is typically observed after 5 days on Marine Agar 2216 plates, when incubated at 25 °C. Colonies are circular and convex with a cream-coloured margin and a raised, dark-brown centre. Older colonies become brown. The temperature range for growth is 5–30 °C, with an optimum of 25–30 °C. The pH range for growth is 7.0–9.0, with an optimum of pH 7.0. Growth is observed with 2–7% (w/v) sea salts. The isoprenoid quinone type is Q-10. Primary cellular fatty acid is 18:1ω7c. Cyclo 19:0 is not observed. Hydrolysis of gelatin, starch, urea, aesculin and *p*-nitrophenol-β-D-galactopyranoside is not observed. Does not reduce nitrate to nitrite. Growth occurs on D-glucose, glycerol, mannitol, sorbitol, starch, D-arabitol and gluconate. Isolated from sea-water samples obtained at a depth of 5 m in Hiroshima Bay, Japan. As well as the type strain, a reference strain (CFPB-A5=LMG 22016=NBRC 100363) is available.

The type strain is CFPB-A9<sup>T</sup> (=LMG 22015<sup>T</sup>=NBRC 100362<sup>T</sup>). Its G+C content is 59.1 mol%.

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