International Journal of Systematic and Evolutionary Microbiology (2002), **52**, 1527–1532 DOI: 10.1099/ijs.0.01911-0

NOTE

Psychromonas kaikoae sp. nov., a novel piezophilic bacterium from the deepest cold-seep sediments in the Japan Trench

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Two strains of obligately piezophilic bacteria were isolated from sediment collected from the deepest cold-seep environment with chemosynthesis-based animal communities within the Japan Trench, at a depth of 7434 m. The isolated strains, JT7301 and JT7304^T, were closely affiliated with members of the genus *Psychromonas* on the basis of 16S rDNA sequence analysis. Hybridization values for DNA–DNA relatedness between these strains and the *Psychromonas antarctica* reference strain were significantly lower than that accepted as the phylogenetic definition of a species. The optimal temperature and pressure for growth of the isolates were 10 °C and 50 MPa and they produced both eicosapentaenoic acid (C20:5ω3) and docosahexaenoic acid (C22:6) in the membrane layer. Based on the taxonomic differences observed, the isolated strains appear to represent a novel obligately piezophilic *Psychromonas* species. The name *Psychromonas kaikoae* sp. nov. (type strain JT7304^T = JCM 11054^T = ATCC BAA-363^T) is proposed. This is the first proposed obligately piezophilic species of the genus *Psychromonas*.

Keywords: *Psychromonas kaikoae*, piezophilic bacteria, Japan Trench, deep-sea, cold-seep

Numerous deep-sea piezophilic bacterial strains have been isolated and characterized in an effort to understand the interaction between the deep-sea environment and its microbial inhabitants (Kato *et al*., 1995, 1996, 1998; Yayanos *et al*., 1979). Piezophilic bacteria, as defined by Yayanos (1995), are characterized by enhanced growth at pressures above 1 atmosphere. All piezophilic bacteria isolated to date fall into the γ-*Proteobacteria* according to phylogenetic classifications based on 16S rRNA sequence information (Kato *et al*., 1995, 1996; DeLong *et al*., 1997; Nogi & Kato, 1999). DeLong *et al*. (1997) reported that 11 cultivated psychrophilic and piezophilic deep-sea bacteria were affiliated with one of five genera within the γ-*Proteobacteria*: *Shewanella*, *Photobacterium*, *Colwellia*, *Moritella* and an unidentified genus. The deep-sea piezophilic species of four of these genera were named *Shewanella benthica* and *Shewanella violacea* (Deming *et al*., 1984; MacDonell & Colwell, 1985; Nogi *et al*., 1998b), *Photobacterium profundum* (Nogi *et al*., 1998c), *Colwellia hadaliensis* (Deming *et al*., 1988) and *Moritella japonica* and *Moritella yayanosii* (Nogi *et al*., 1998a; Nogi & Kato, 1999). The fifth genus, which is as yet undescribed, was called the CNPT-3 group (DeLong *et al*., 1997). Members of the genera *Shewanella*, *Photobacterium* and *Moritella* are not unique to deep-sea marine environments and most of these isolates are not obligately piezophilic strains. *Colwellia hadaliensis* strain BNL-1^T (Deming *et al*., 1988) and *Moritella yayanosii* strain DB21MT-5^T (Nogi & Kato, 1999) were reported as obligately piezophilic strains within this group. However, identification of piezophilic species belonging to the fifth piezophilic genus, the CNPT-3 group, remains necessary to understand the taxonomic position of this group of organisms from the deep-sea environment.

In this paper, results of taxonomic studies on obligately piezophilic strains isolated from the deepest cold-seep environment in the Japan Trench are described. Several lines of evidence indicate that two of these isolates, strains JT7301 and JT7304^T, represent a novel

Abbreviations: ASW, artificial sea water; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA sequence of *Psychromonas kaikoae* JT7304^T is AB052160.

species within the genus *Psychromonas*. The type species of the genus *Psychromonas*, *Psychromonas antarctica*, was isolated as an aerotolerant anaerobic bacterium from a high-salinity pond on Antarctica (Mountfort *et al*., 1998). This strain did not display piezophilic properties. Our results suggest that strains JT7301 and JT7304^T can be classified as members of a novel species, proposed as *Psychromonas kaikoae* sp. nov. This is the first report that identifies a piezophilic species belonging to the genus *Psychromonas*.

The obligately piezophilic *Psychromonas* strains JT7301 and JT7304^T were isolated from sediment of the deepest cold-seep environment with chemosynthesis-based animal communities such as *Maorithyas hadalis* (Fujikura *et al*., 1999), obtained using sterilized mud samplers on the unmanned submersible *KAIKO* from the Japan Trench (40° 2·8′ N, 144° 16·6′ E) at a depth of 7434 m. The reference strain used in this study, *Psychromonas antarctica* DSM 10704^T, was obtained from the DSMZ. These bacteria were maintained on marine agar 2216 (Difco). *Psychromonas antarctica* was grown at 12 °C and atmospheric pressure; the isolated piezophilic strains were grown at 10 °C and 50 MPa. High-pressure cultivation was achieved using a liquid hydraulic system. Piezophilic bacteria were cultivated in a plastic bag containing liquid medium in a pressure vessel made of stainless steel (SUS304). If necessary, oxygen-saturated fluorinert (FC-72; Sumitomo-3M) was added to supply oxygen to the cultures (20% total volume). This method was performed according to the procedure reported previously (Kato *et al*., 1994; Yanagibayashi *et al*., 1999).

Optimal pressure and temperature for growth were measured by monitoring the OD and cell numbers were determined by microscopy. These tests were performed anaerobically in marine broth 2216 under different pressure and temperature conditions.

Physiological tests were performed by slight modifications of the general procedures described by Barrow & Feltham (1993) and DeLong *et al*. (1997). All high-pressure physiological tests were performed in tandem with uninoculated blank controls according to the following procedure. Acid production from sugars was assessed using modified OF medium (Hugh & Leifson, 1953) containing 0·5× artificial sea water (ASW; 1×ASW consists of 3% NaCl, 0·07% KCl, 1·08% $MgCl_2.6H_2O$, 0·54% $MgSO_4.7H_2O$, 0·1% $CaCl_2.2H_2O$), 0·1% $(NH_4)_2HPO_4$, 0·02% yeast extract, 0·1% Na_2CO_3, 1% sugar, 0·5% low-melting-point agar and 0·003% bromthymol blue (the pH was adjusted to 7·1 at 20 °C). Fermentation test cultures were inoculated as stabs and sealed with molten OF medium. Oxidization test cultures were inoculated as stabs and covered with oxygen-saturated fluorinert. After capping the tube, it was sealed with Parafilm and incubated at 50 MPa and 10 °C for several days. After decompression, the stabs were examined for growth and acid production. Any gases produced by the bacteria were dissolved into medium under pressure conditions (Nogi & Kato, 1999). Physiological tests under high-pressure conditions, examining hydrogen sulfide production from thiosulfate, production of indole, gelatinase activity, oxidase and catalase production, were performed according to methods described previously (Nogi & Kato, 1999).

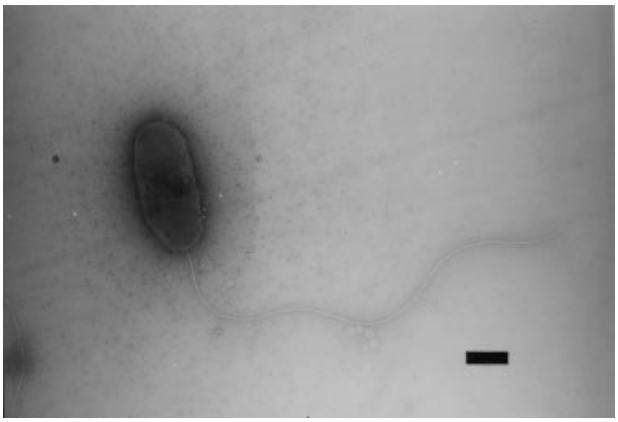

Fig. 1. Transmission electron micrograph of a negatively stained cell of strain JT7304^T. Bar, 0·5 μm.

Cells of the isolated deep-sea strain JT7304^T were found to be Gram-negative rods, 2–4 μm long and 0·8–1·0 μm wide, motile by means of a single unsheathed polar flagellum (Fig. 1). This strain was unable to grow at atmospheric pressure and the cells displayed unusual shapes, appearing as elongated forms at 4 °C and atmospheric pressure. However, the strain grew well in pressure vessels under hydrostatic pressures of 10–70 MPa at 4–15 °C; the optimal pressure and temperature for growth were 50 MPa and 10 °C (Fig. 2a). The reference strain, *Psychromonas antarctica*, was not able to grow under such high-pressure conditions (Fig. 2b). Characteristics of the isolated piezophilic *Psychromonas* strains and the reference strains are shown in Table 1. *Psychromonas antarctica* grew well under aerobic or anaerobic conditions in marine broth 2216 and the characteristics of this species were tested under aerobic conditions. Strains JT7301 and JT7304^T are facultatively anaerobic chemo-organotrophs, displaying both respiratory and fermentative types of metabolism. Other characteristics of JT7301 and JT7304^T were as follows. Acid, but not gas, was produced from cellobiose, D-fructose, D-galactose, D-glucose, maltose, D-mannitol, D-mannose, sucrose and D-trehalose. The catalase and cytochrome oxidase test results were positive, gelatin was hydrolysed, nitrate was reduced to nitrite, but nitrite was not reduced. Test results for amylase, H_2S production and indole production were negative. The following compounds were not utilized: L-arabinose, glycerol, *myo*-inositol, D-lactose, D-raffinose, L-rhamnose, D-sorbitol and xylose. The G+C content of the DNA was 43·8 mol%. The major isoprenoid quinone was Q-8 (ubiquinone-8). The reference strain, *Psychromonas an-*

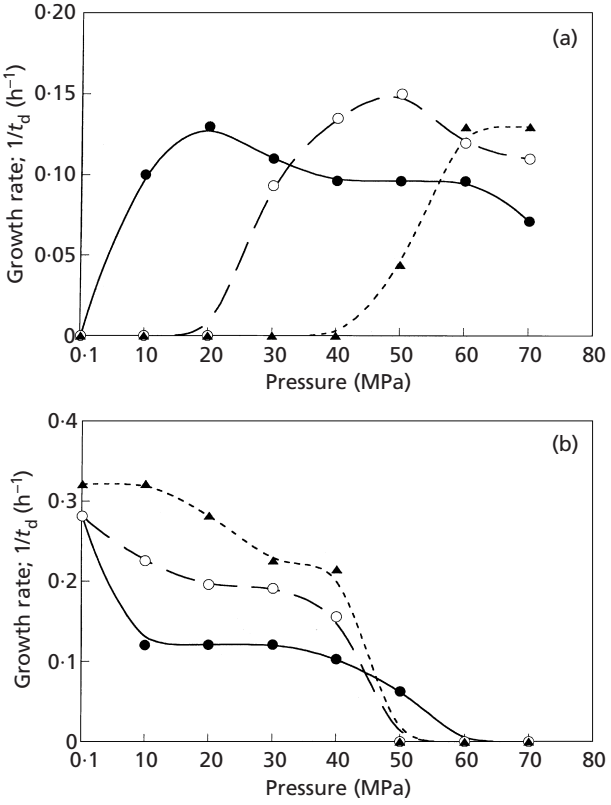

Fig. 2. Growth profiles of the piezophilic bacterium *Psychromonas kaikoae* sp. nov. strain JT7304^T (a) and *Psychromonas antarctica* (b) at several pressures and at temperatures of 4 (●), 10 (○) and 15 (▲) °C. Growth rate is shown as $1/t_d$, where t_d is the doubling time (h).

tarctica, shared most physiological characteristics with JT7304^T, including the carbohydrate-utilization profile. However, unlike the type species of *Psychromonas*, the strains from the Japan Trench reduced nitrate and they were unable to grow at atmospheric pressure.

16S rRNA gene sequences were obtained by direct sequencing of PCR-amplified DNA, as described previously (Kato *et al.*, 1998). Nucleotide substitution rates (K_{nuc}; Kimura, 1980) were determined and a distance-matrix tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) using the program CLUSTAL W (Thompson *et al.*, 1994). Alignment gaps and unidentified base positions were not taken into consideration in the calculations. The topology of the phylogenetic tree was evaluated by performing bootstrap analysis with 1000 bootstrapped trials. The results of phylogenetic analyses based on 16S rDNA sequence information support the conclusions described below and further clarify the taxonomic and phylogenetic position of the novel isolates among members of the genus *Psychromonas* and related genera. The results of the phylogenetic analyses are shown in Fig. 3. Strains JT7301 and JT7304^T fall into the genus *Psychromonas* and are closely related to the psychrophilic type species, *Psychromonas antarctica*, and the unidentified piezophilic strain, CNPT-3. Strains JT7301 and JT7304^T were closely related to and on the same branch as strain CNPT-3, with sufficient distance between each species, and belong in a cluster with the previously described CNPT-3 group. However, inclusion of strain CNPT-3 in the genus *Psychromonas* is problematic because of its spirillum morphology (DeLong *et al.*, 1997). For analysis of relatedness, DNA–DNA hybridization was carried out at 40 °C for 3 h and measured fluorometrically by the method of Ezaki *et al.* (1989). The results of DNA–DNA hybridization analysis comparing strain JT7301, JT7304^T and *Psychromonas antarctica* are as follows. A high level of DNA–DNA relatedness (92–100 %) was observed between the isolated piezophilic strains JT7301 and JT7304^T. The similarity

Table 1. Phenotypic characteristics of isolates JT7301 and JT7304^T and *Psychromonas antarctica*

Both species were Gram-negative rods, but did not form spores. They were motile by means of a polar flagellum. All strains were positive for catalase, oxidase and hydrolysis of gelatin. The major isoprenoid quinone type was Q-8. Acid was produced from cellobiose, D-fructose, D-galactose, D-glucose, maltose, D-mannitol, sucrose and D-trehalose. All strains were negative for hydrolysis of starch, production of H_2S and indole, nitrite reduction, gas production from carbohydrates and acid production from L-arabinose, *myo*-inositol, D-lactose, D-raffinose, L-rhamnose, D-sorbitol and xylose. +, Positive; −, negative; w, weakly positive after 3 weeks.

Characteristic	*Psychromonas kaikoae* JT7301 and JT7304^T	*Psychromonas antarctica* DSM 10704^T
Optimum growth conditions:		
Temperature (°C)	10	15
Pressure (MPa)	50	0·1–10
G+C content (mol %)	43·1–43·8	43·0
Nitrate reduction to nitrite	+	−
Acid production from:		
Glycerol	w	+
D-Mannose	+	w

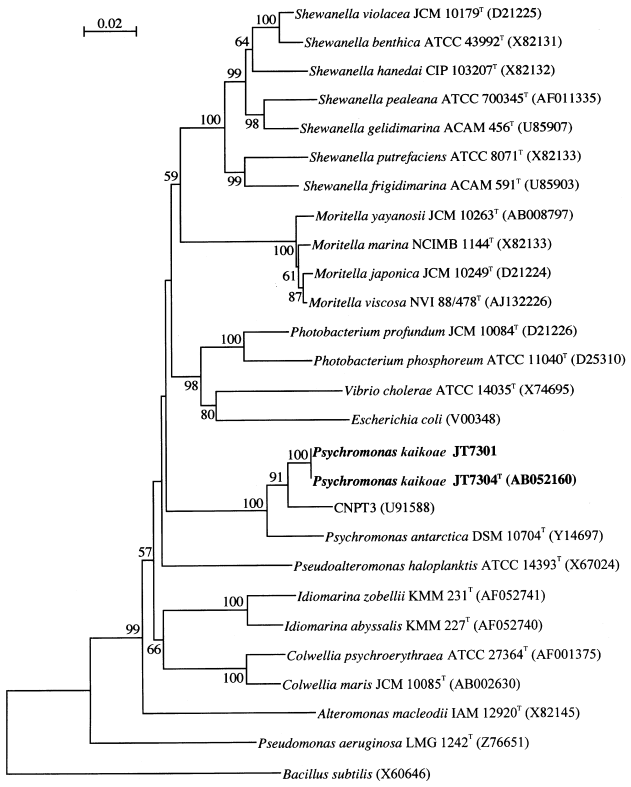

Fig. 3. Phylogenetic tree showing the relationships of strains JT7301 and JT7304^T within the *γ-Proteobacteria*. The tree was constructed by the neighbour-joining method and based on 16S rRNA gene sequences. Bar, 0·02 nucleotide substitutions per site. Bootstrap values were calculated from 1000 trees.

between either JT7301 or JT7304^T and the *Psychromonas antarctica* reference strain was less than 27%. This is significantly lower than that accepted as the phylogenetic definition of a species (Wayne *et al.*, 1987). This and other results in Table 1 and Fig. 3 suggest that strains JT7301 and JT7304^T represent a novel *Psychromonas* species.

The whole-cell fatty acid compositions of the piezophilic strains JT7301 and JT7304^T and reference strains are shown in Table 2. The major fatty acids in strains JT7301 and JT7304^T were C14:0 (tetradecanoic acid), C14:1 (tetradecenoic acid), C16:0 (hexadecanoic acid) and C16:1 (hexadecenoic acid). There was a low level of similarity between this fatty acid profile and that of the *Psychromonas antarctica* type strain. For example, the predominant components in the fatty acid profile of the *Psychromonas antarctica* type strain differed from those in the profile of strains JT7301 and JT7304^T, which contained substantial amounts of C14:0 and small amounts of the long-chain unsaturated fatty acids C20:5ω3 [eicosapentaenoic acid (EPA)] and C22:6 [docosahexaenoic acid (DHA)].

On the basis of the phenotypic, genotypic and phylogenetic data, it is logical to conclude that the deep-sea

Table 2. Fatty acid composition of *Psychromonas* strains and piezophilic bacteria

Values are percentages of total fatty acids. Strains: 1, *Psychromonas kaikoae* JT7301; 2, *Psychromonas kaikoae* JT7304^T; 3, *Psychromonas antarctica* DSM 10704^T; 4, *Shewanella benthica* ATCC 43992^T; 5, *Shewanella violacea* JCM 10179^T; 6, *Moritella japonica* JCM 10249^T; 7, *Moritella yayanosii* JCM 10263^T; 8, *Photobacterium profundum* JCM 10084^T. tr, Trace; empty cells, fatty acid not detected.

Fatty acid	1	2	3	4	5	6	7	8
11:0				tr	3			
12:0	1	1	1	2	4			2
13:0					tr			
14:0	7	6		14	5	18	15	3
15:0	1	1			6	1	1	1
16:0	13	15	24	14	14	21	13	9
17:0				tr	1			
18:0								1
iso-13:0				5	7			2
iso-14:0				tr				4
iso-15:0				11	13			2
iso-16:0								15
14:1ω7*t*	10	10	8		1	2	6	3
16:1ω7*c*	53	52	58	31	16	45	48	31
16:1ω9*c*	3	2			tr	5	5	
17:1ω6*c*					1			
17:1ω8*c*				tr	2			
18:1ω7*c*	2	2	3	2	4	2	1	9
18:1ω9*c*					tr			
20:5ω3	2	2		16	14			13
22:6	3	2				6	11	
3OH-12:0	3	2		1	1			5
3OH-iso-13:0				5	8			
3OH-14:0	3	4	6					

isolates studied are members of the genus *Psychromonas* and represent a novel species within this genus. The name *Psychromonas kaikoae* sp. nov. is proposed, with strain JT7304^T (= JCM 11054^T = ATCC BAA-363^T) as the type strain.

Several deep-sea piezophilic bacterial strains have been isolated and characterized (Kato *et al.*, 1995, 1996, 1998; Yayanos *et al.*, 1979) and cultivated psychrophilic and piezophilic deep-sea bacteria are affiliated with one of five genera within the *γ-Proteobacteria*: *Shewanella*, *Photobacterium*, *Colwellia*, *Moritella* and an undescribed genus (DeLong *et al.*, 1997). The first obligately piezophilic bacterium isolated from the Mariana Trench was a member of the genus *Colwellia*, strain MT41 (Yayanos *et al.*, 1981; DeLong *et al.*, 1997). Since then, three obligately piezophilic bacterial strains have been isolated, designated *Colwellia hadaliensis* BNL-1^T (Deming *et al.*, 1988), *Shewanella benthica* DB21MT-2 (Kato *et al.*, 1998; Nogi & Kato, 1999) and *Moritella yayanosii* DB21MT-5^T (Kato

et al., 1998; Nogi & Kato, 1999). A novel obligately piezophilic species within the fifth genus, *Psychromonas*, is reported here, based on the results of phylogenetic analysis of 16S rDNA sequences and several other taxonomic properties described in this paper.

Mountfort *et al*. (1998) reported that *Psychromonas antarctica* is an aerotolerant bacterium. However, according to our results, *Psychromonas antarctica* should be categorized as facultatively anaerobic, because this bacterium grew well under aerobic conditions in marine broth 2216, the catalase and cytochrome oxidase test results were positive and the major isoprenoid quinone was Q-8 (Table 1).

It has been reported previously that the deep-sea piezophilic species *Shewanella benthica* and *Shewanella violacea* are closely related to cold-adapted *Shewanella* strains isolated from polar regions (Kato & Nogi, 2001). Maruyama *et al*. (2000) also reported that *Psychrobacter pacificensis*, isolated from Japan Trench sea water at a depth of 5000–6000 m, was taxonomically similar to the Antarctic isolates *Psychrobacter immobilis*, *Psychrobacter gracincola* and *Psychrobacter frigidicola* (Maruyama *et al*., 2000). They concluded that the occurrence of *Psychrobacter* strains in cold sea water deep in the Japan Trench and at the surface of the Antarctic sea suggests that bacterial habitation of the deep sea and their evolution have been influenced by the global deep-ocean circulation linked to the sinking of cooled sea water in polar regions (Schmitz, 1995). In the case of the genus *Psychromonas*, *Psychromonas antarctica* was isolated from sediment in the Antarctic (Mountfort *et al*., 1998) and it is possible that the presence of piezophilic *Psychromonas* strains JT7301 and JT7304^T at the bottom of the Japan Trench is due to ocean circulation.

The piezophilic and psychrophilic *Shewanella* and *Photobacterium* strains produce EPA (C20:5ω3) and *Moritella* strains produce DHA (C22:6). Generally, piezophilic and psychrophilic bacteria produce only one or other of these long-chain polyunsaturated fatty acids. *Psychromonas kaikoae* strains JT7301 and JT7304^T are unusual piezophilic strains in that they produce both EPA and DHA.

Emended description of the genus *Psychromonas* (Mountfort *et al*. 1998)

Psychromonas (Psy.chro.mo′nas. Gr. adj. *psychros* cold; Gr. fem. n. *monas* a unit; N.L. n. *Psychromonas* a cold monad).

Facultatively anaerobic. Gram-negative, rod- to oval-shaped and motile with a polar flagellum. Temperature range for growth is below 22 °C. Carbohydrates are the principal energy source for growth. Catalase and cytochrome oxidase test results are positive. The major isoprenoid quinone is Q-8. Predominant cellular fatty acids are C14:1, C16:0 and C16:1. The type species is *Psychromonas antarctica*.

Description of *Psychromonas kaikoae* sp. nov.

Psychromonas kaikoae (kai.ko′ae. N.L. fem. gen. n. *kaikoae* of *KAIKO*, the unmanned submersible that collected the samples from which the organism was isolated).

Cells are Gram-negative rods, 2·0–4·0 × 0·8–1·0 μm, motile by means of a single unsheathed polar flagellum. Halophilic, psychrophilic and piezophilic. Optimal growth occurs at an NaCl concentration of about 3 %. No growth occurs in the absence of NaCl. Optimal temperature and pressure for growth are 10 °C and 50 MPa. No growth occurs at atmospheric pressure. Facultatively anaerobic chemo-organotroph, having both respiratory and fermentative types of metabolism. Catalase and cytochrome oxidase test results are positive, gelatin is hydrolysed, nitrate is reduced to nitrite, but nitrite is not reduced. Amylase, H_2S production and indole production are negative. G+C content of the DNA is about 43·8 mol %. The major isoprenoid quinone is Q-8. Predominant cellular fatty acids are C14:0, C14:1, C16:0 and C16:1; small amounts of both C20:5ω3 and C22:6 are present. Other characteristics of this strain are shown in Table 1. The type strain is strain JT7304^T (= JCM 11054^T = ATCC BAA-363^T).

Acknowledgements

We are very grateful to the *KAIKO* operation team and the crew of MS *Kairei* for helping us to collect the deep-sea samples. We also thank Mr M. Mori and S. Hosoya for technical assistance in isolating strains JT7301 and JT7304^T, Mr Uematsu for operating the electron microscopic system and Dr W. R. Bellamy for assistance in editing the manuscript.

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