

Prototheca cutis sp. nov., a newly discovered pathogen of protothecosis isolated from inflamed human skin

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A strain of a novel pathogenic, achlorophyllic alga belonging to the genus *Prototheca* was isolated from the inflamed skin of a patient with protothecosis in a Japanese hospital. The pathogen was detected and isolated in biopsy specimens by histopathology and culture-based examination. Analyses of the nuclear 18S rDNA gene and 26S rDNA gene D1/D2 domain sequences and chemotaxonomic studies indicated that this strain represents a novel species with a close phylogenetic relationship to *Prototheca wickerhamii* and *Auxenochlorella protothecoides*. This strain grew well at 28–30 °C, showed slow and weak growth at 37 °C, and no growth at 40 °C. This strain grew in vitamin-free medium and assimilated acetate (pH 5.1), L-arabinose and soluble starch as a carbon source. The taxonomic description of *Prototheca cutis* sp. nov. is proposed (type strain JCM 15793^T = CBS 11262^T = DSM 22084^T) as a pathogen of dermatitis.

Protothecosis is a sporotrichosis-like dermatitis in humans and animals caused by infection with achlorophyllic algae of the genus *Prototheca*. The genus *Prototheca* consists of achlorophyllous algae closely related to the green algae of the genus *Chlorella* (Pore, 1998). Species of the genus *Prototheca* exist in the environment as ubiquitous detritus inhabitants and contaminants of various substrates. General protothecosis is caused in humans by *Prototheca wickerhamii* and in domestic animals by *Prototheca zopfii*. The general symptom is dermatitis or bovine mastitis, and mortal cases are extremely rare. On the other hand, *P. wickerhamii* is a serious pathogen which rarely causes meningitis (Kaminski *et al.*, 1992). Their roles as pathogens of human diseases are largely unknown. However, the incidence of infections caused by unusual organisms is likely to increase with the increasing numbers of immunocompromised individuals throughout the world (Lass-Flörl & Mayr, 2007).

In this study, we characterized a pathogenic strain isolated from inflamed skin caused by protothecosis in a patient in a Japanese hospital. Phenotypic, chemotaxonomic, and

phylogenetic analyses indicated the strain to be affiliated with the genus *Prototheca*. The data obtained also suggested that the isolate represents a novel species as a pathogen of dermatitis.

Most of the morphological, biochemical, and physiological characteristics were examined using the methods described by Yarrow (1998). Skin samples obtained by biopsy were subjected to both histopathological and microbiological examinations. For histopathological examination, the sample was digested in diastase and paraffin-embedded pathological specimens were subjected to periodic acid-Schiff staining. For microbiological examination, samples were inoculated onto Sabouraud dextrose agar (SDA), and incubated at 35 °C. Assimilation pattern-based identification of the isolate was performed with API 20C AUX V4.0 strips (bioMérieux). Fatty acid methyl esters were determined by TechnoSuruga Laboratory (Shizuoka, Japan). Yeast nitrogen base with amino acids and ammonium sulfate, vitamin free (Y2035; United States Biological), was used as vitamin-free medium.

Genomic DNA from the isolate was extracted and purified according to the protocol recommended for the FTA Classic Card (Whatman). DNA fragments covering the nuclear 18S rDNA gene and the 26S rDNA gene D1/D2 domain were amplified by using primers for the 18S rDNA gene (SSU-F1 and SSU-R1) and the D1/D2 domain (28SF1 and 635), respectively, as described previously (Sugita

The GenBank/EMBL/DDBJ accession number for the 18S rDNA gene and 26S rDNA gene D1/D2 domain sequences of strain JCM 15793^T are AB470468 and AB470469, respectively.

Larger versions of Figures 1 and 2, and a phylogenetic tree based on 26S rRNA gene D1/D2 domain sequences are available with the online version of this paper.

et al., 2003; White *et al.*, 1990; Xiao *et al.*, 2004). DNA sequencing was performed with an Applied Biosystems model 310 sequencer. A neighbour-joining tree (Saitou & Nei, 1987) was reconstructed by using MEGA4 (Tamura *et al.*, 2007). Data consistency was tested by bootstrapping the alignments 1000 times with corrections for multiple substitutions.

The patient was a 57-year-old male civil service employee. He does not touch the soil or domestic animals in daily life. The chief condition was a left antebrachial ulcer. He had past histories of diverticulitis (at 35 years), chronic persistent HCV hepatitis (from 46 years to present), urothelial carcinoma (at 48 years; grade I, treated successfully by endoscopic surgery), diffuse large B-cell malignant lymphoma (at 51 years; achieved complete remission with chemotherapy), and psoriasis vulgaris (at 51–57 years; treated by prednisolone by mouth, 8–15 mg day⁻¹). In April 2008, the patient noticed ulcer formation in his forearm without any injury. He visited Asahi General Hospital (Chiba, Japan) in May because the ulcer had not subsided. The affected area showed cellulitis-like dermatitis with ulcer (70 × 25 mm). Biopsy was performed under a suspected diagnosis of sporotrichosis (Fig. 1 and Supplementary Fig. S1, available in IJSEM Online). The case was suspected to be protothecosis because a yeast strain was isolated from the biopsy sample and cells typical of the genus *Prototheca* were observed on histopathological examination (Fig. 2 and Supplementary Fig. S2). Therefore, itraconazole (by mouth, 200 mg day⁻¹) was prescribed from June. No histopathological characteristics of psoriasis vulgaris were observed. In July, a second biopsy was performed because the dermatitis had improved but rubor and some small abscesses remained. A yeast-like strain was isolated again and cells typical of the genus *Prototheca* were still observed. Continuous treatment with itraconazole induced remission by October. The yeast-like isolates from May and July were subjected to an assimilation-based identification test by API 20C and both strains were



Fig. 1. The symptom that was caused by infection with strain JCM 15793^T. Cellulitis-like dermatitis with ulcer (70 × 25 mm) was observed on the left forearm.

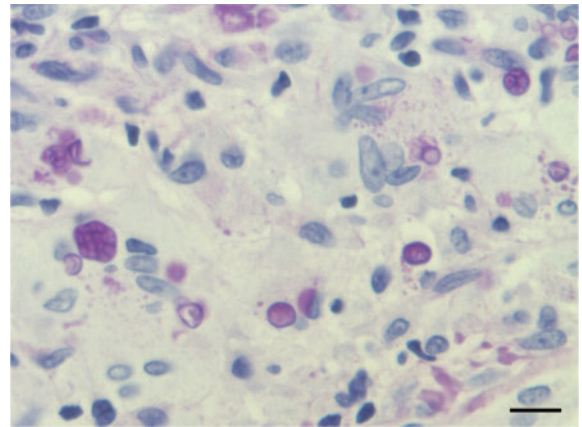


Fig. 2. Histopathological image of biopsy specimen. Bar, 10 μm.

identified as *P. wickerhamii* with a probability of 99.9 % (API profile: 6240040).

As described in the species description, the main characteristics of strain JCM 15793^T (July isolate) did not correspond to those of *P. wickerhamii* but to a novel species. The colonies could grow in the light (3–30 μmol photons m⁻² s⁻¹) or dark and were white to ivory with no relation to the light or dark growth conditions. Fig. 3 shows a micrograph of a culture of strain JCM 15793^T. The strain grew well at 28–30 °C, showed slow and weak growth at 37 °C, and did not grow at 40 °C. Strain JCM 15793^T showed slow and weak growth in vitamin-free medium. The percentages of fatty acid methyl esters were as follows: 2.04 % C_{14:0}, 0.51 % C_{15:0}, 0.62 % C_{16:1ω7c}, 19.7 % C_{16:0}, 0.62 % C_{16:1}, 0.65 % C_{17:1ω8c}, 0.79 % C_{17:0}, and 4.55 % C_{18:0}.

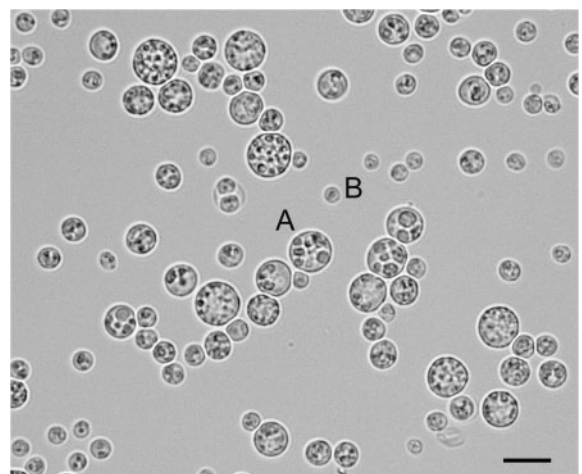


Fig. 3. Culture of strain JCM 15793^T photographed after an incubation period of 3 days at 30 °C on Sabouraud dextrose agar plates. A, Sporangia; B, released sporangiospores. Bar, 10 μm.

Phylogenetic trees of strain JCM 15793^T based on 18S rDNA gene and 26S rDNA gene D1/D2 domain sequences are shown in Fig. 4 and Supplementary Fig. S3, respectively. These two neighbour-joining trees suggested that strain JCM 15793^T represented a novel species. The 18S rDNA gene sequence of JCM 15793^T showed 92.2 and 90.5% similarity to those of *P. wickerhamii* SAG 263-11 and *Auxenochlorella protothecoides* SAG 211-7a^T, respectively. The 26S rDNA gene D1/D2 region of JCM 15793^T showed 86.5 and 82.2% similarity to those of *P. wickerhamii* SAG263-11 and *A. protothecoides* SAG 211-7a^T, respectively.

The infection in the present case was an ulcer-forming cellulitis-like dermatitis related to systemic prednisolone or some primary disease. It was not a systemic but superficial infection; however, it should be noted that the novel species could not be distinguished from superficial and systemic infection-causing *P. wickerhamii* by the conventional identification system (Mohabeer *et al.*, 1997).

Strain JCM 15793^T clustered with the genus *Prototheca*, but was different from the recognized species (Fig. 4 & Supplementary Fig. S3). There have been previous reports of fatty acid methyl ester analysis of the genus *Prototheca*, but comparison with the present study was not possible due to the different methods used (Roesler *et al.*, 2006; Sud & Feingold, 1979). However, there are no species closely related to JCM 15793^T in The Sherlock Microbial Identification System (MIDI). Since the related species *A. protothecoides* (Fig. 4) has chlorophyll, the cultures become green in the light (Kalina & Punčochářová, 1987). The

culture of JCM 15793^T was ivory–white in the light, and therefore did not belong to the genus *Auxenochlorella*. The typical API profile of *P. wickerhamii* is 6040040. Strain JCM 15793^T differed from *P. wickerhamii* in L-arabinose assimilation, but was classified as *P. wickerhamii* showing a non-specific reaction. Furthermore, the results of acetate (pH 5.1) and soluble starch assimilation were also different, but API does not support these. Therefore, it was not possible to classify these two species by using the API system. With the exception of *Prototheca blaschkeae*, members of the genus *Prototheca* cannot grow in vitamin-free medium and cannot assimilate soluble starch (Pore, 1998). These two characteristics have not been described in *P. blaschkeae* (Roesler *et al.*, 2006). The recognized species of the genus *Prototheca* cannot assimilate L-arabinose (Pore, 1998; Roesler *et al.*, 2006). As strain JCM 15793^T was different from the recognized species in these characteristics, it was suggested to be a novel species. Strain JCM 15793^T was identified as a novel pathogen involved in protothecosis. This strain was not identified with the commercial kit based on carbon assimilation, and was shown to represent a novel species by gene analysis. Therefore, the importance of gene diagnosis will likely increase in the future. Taxonomically, it is desirable that a description of a new species be based on more than one isolate. However, as our novel species grew in vitamin-free medium and assimilated acetate (pH 5.1), L-arabinose and soluble starch, this micro-organism appears to be significant as a member of the genus *Prototheca*. The infection by this isolate was of a cuticle and was not systemic. This is a symptom unlike *P. wickerhamii*. Furthermore, information

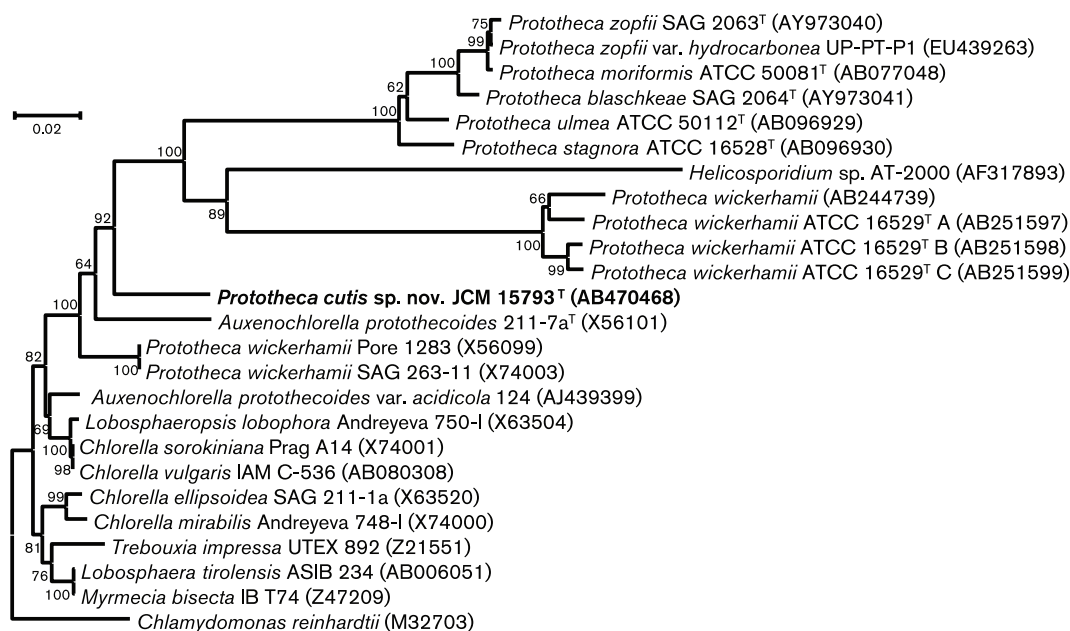


Fig. 4. Phylogenetic tree drawn from neighbour-joining analysis of 18S rDNA gene partial sequences, depicting the relationships of strain JCM 15793^T with closely related species. Bootstrap percentages over 60% from 1000 bootstrap replicates are shown. Bar, 0.02 substitutions per nucleotide position. Outgroup: *Chlamydomonas reinhardtii* (M32703).

regarding this novel pathogen will help in the treatment of protothecosis. Therefore, we propose a novel species based on this single isolate. In addition, *P. wickerhamii* formed two phylogenetic clusters in this study (Fig. 4 and Supplementary Fig. S3). *P. wickerhamii* is a historic species that was described 50 years ago and has not yet been subdivided (Pore, 1998; Tubaki & Soneda, 1959). Therefore, the possibility to re-examine the classification of strains within this species in the future is suggested.

Latin diagnosis of *Prototheca cutis* Satoh et Makimura sp. nov.

In liquido YM post dies 3 ad 30 °C, cellulae vegetativae, ovoideae aut globosae, 2.0–8.0 µm, singulae, binae. Sedimentum formatur. In SDA post dies 3 ad 30 °C, cultura albus ad eboris, glabra, butyracea vel viscida, margine glabra. Assimilat sic: glucosum, D-galactosum, saccharosum (exiguae), maltosum (exiguae), cellobiosum (exiguae), D-trehalosum, lactosum, inulin (exiguae), amyllum solubile (exiguae), L-arabinosum, D-glucosaminum (exiguae), ethanolum, glycerolum, acidum DL-lacticum, 1-propanolum (exiguae), acidum aceticum (pH 5.1), fructosum, et mannosum; non respondent, L-sorbosum, melibiosum, D-raffinose, D-melezitosum, D-xylosum, D-arabinosum, ribosum, L-rhamnosum, N-acetyl-D-glucosaminum, methanolum, erythritolum, ribitolum, galactitolum, D-mannitolum, D-glucitolum, methyl α-D-glucosidum, salicinum, acidum gluconicum, acidum succinicum, inositolum, hexadecanum, nec 2-ketogluconatum. Ammonium sulfatum et cadaverinum (exiguae), assimulantur at non natrium nitrosum, kalium nitricum, ethylaminum, nec L-lysinum. Arginine non assimilantur. Ad crescentiam vitaminum non necessarium est (exiguae). Augmentum in 28 °C–30 °C, crescit in 37 °C, non crescit in 40 °C. Typus stirps JCM 15793^T ex auris proluerit homine, Japonia, isolata est. In collectionibus culturarum quas Japan Collection of Micro-organisms, Wako, Saitama, sustentant, no. JCM 15793^T, Centraalbureau voor Schimmelcultures, Delphi Batavorum, sustentant, no. CBS 11262^T, et Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany, sustentant, no. DSM 22084^T deposita est.

Description of *Prototheca cutis* Satoh & Makimura sp. nov.

Prototheca cutis (cu'tis. L. gen. f. n. *cutis* of the skin, isolated from the skin of a human patient).

After 3 days in YM broth at 30 °C, cells are ovoid to globose, 2.0–8.0 µm, and single. Sediment is formed. After 3 days on Sabouraud dextrose agar at 30 °C, the streak culture is butyrous to viscous, white to ivory, smooth and glistening with an entire margin. The following carbon compounds are assimilated: glucose, D-galactose, sucrose (weak), maltose (weak), D-cellobiose (weak), D-trehalose, lactose (weak), inulin (weak), soluble starch, L-arabiose, D-glucosamine (weak), ethanol, glycerol, DL-lactate, 1-propanol (weak), acetate (pH 5.1), fructose and mannose.

The following are not assimilated: L-sorbose, melibiose, D-raffinose, D-melezitose, D-xylose, D-arabinose, ribose, L-rhamnose, N-acetyl-D-glucosamine, methanol, erythritol, ribitol, galactitol, D-mannitol, sorbitol, α-methyl-D-glucoside, salicin, D-gluconate, succinate, citrate, inositol, hexadecane and 2-keto-D-gluconate. Ammonium sulfate and cadaverine (weak) are utilized as sole sources of nitrogen; sodium nitrite, potassium nitrate, ethylamine and L-lysine are not utilized. Arginine is not utilized. Growth in vitamin-free medium is positive, slow and weak. Optimum growth temperature is 28–30 °C; growth is positive at 37 °C, negative at 40 °C.

The type strain, JCM 15793^T, was isolated from inflamed skin of a male patient at Asahi General Hospital (Chiba, Japan). This strain has been deposited in the Japan Collection of Micro-organisms, Wako, Saitama, as JCM 15793^T, the Yeast Division of the Centraalbureau voor Schimmelcultures, Delft, the Netherlands, as CBS 11262^T, and the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany, as DSM 22084^T.

Acknowledgements

This study was supported in part by Health Science Research Grants for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labor, and Welfare of Japan (K. M.).

References

- Kalina, T. & Punčochářová, M. (1987). Taxonomy of the subfamily *Scotiellocoystoideae* Fott 1976 (Chlorellaceae, Chlorophyceae). *Arch Hydrobiol* 73, 473–521.
- Kaminski, Z. C., Kapila, R., Sharer, L. R., Kloser, P. & Kaufman, L. (1992). Meningitis due to *Prototheca wickerhamii* in a patient with AIDS. *Clin Infect Dis* 15, 704–706.
- Lass-Flörl, C. & Mayr, A. (2007). Human protothecosis. *Clin Microbiol Rev* 20, 230–242.
- Mohabeer, A. J., Kaplan, P. J., Southern, P. M., Jr & Gander, R. M. (1997). Algaemia due to *Prototheca wickerhamii* in a patient with myasthenia gravis. *J Clin Microbiol* 35, 3305–3307.
- Pore, R. S. (1998). *Prototheca* Krüger. In *The Yeasts, a Taxonomic Study*, 4th edn, pp. 883–887. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.
- Roesler, U., Möller, A., Hensel, A., Baumann, D. & Truyen, U. (2006). Diversity within the current algal species *Prototheca zopfii*: a proposal for two *Prototheca zopfii* genotypes and description of a novel species, *Prototheca blaschkeae* sp. nov. *Int J Syst Evol Microbiol* 56, 1419–1425.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4, 406–425.
- Sud, I. J. & Feingold, D. S. (1979). Lipid composition and sensitivity of *Prototheca wickerhamii* to membrane-active antimicrobial agents. *Antimicrob Agents Chemother* 16, 486–490.
- Sugita, C., Makimura, K., Murai, Y., Yamaguchi, H. & Nagai, N. (2003). A case of pulmonary aspergilloma molecular biological identification and typing of the isolates from antemortem sputa and autopsy fungus ball. *Mycoses* 46, 149–152.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.

Tubaki, K. & Soneda, M. (1959). Cultural and taxonomic studies on *Prototheca. Nagaoa* (*Mycol J Nagao Inst Tokyo*) **6**, 25–34.

White, T. J., Bruns, T., Lee, S. & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: a Guide to Methods and Applications*, pp. 315–322.

Edited by M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White. New York: Academic Press.

Xiao, L., Fayer, R., Ryan, U. & Upton, S. J. (2004). *Cryptosporidium* taxonomy: recent advances and implications for public health. *Clin Microbiol Rev* **17**, 72–97.

Yarrow, D. (1998). Methods for the isolation, maintenance and identification of yeasts. In *The Yeasts, a Taxonomic Study*, 4th edn, pp. 77–100. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.