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.

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In order to clarify the intraspecies taxonomic position of the non-photosynthetic algal species *Prototheca zopfii*, as well as the aetiology of bovine mammary protothecosis, a selection of *P. zopfii* strains isolated from clinical cases of protothecal mastitis and from various environmental habitats was characterized using a polyphasic molecular approach. Based on sequence analysis of the 18S rRNA gene, which showed distinct differences between the three currently known biotypes or 'variants', specific oligonucleotides were designed and used in biotype-specific PCRs. Furthermore, the pattern of cellular fatty acids was evaluated. Typing by means of these techniques revealed that the previously defined biotypes of *P. zopfii* were clearly different. Based on sequence analysis, the pattern of fatty acids and physiological characteristics, it is proposed that biotype 3 should be reclassified as representing a novel species, *Prototheca blaschkeae* sp. nov. (type strain, RZIII-3^T = SAG 2064^T). Furthermore, it is proposed that *P. zopfii* merits reclassification as a species comprising at least two genotypes that in future could probably be considered to represent two subspecies.

Colourless algae of the genus *Prototheca* of the family Chlorellaceae have been identified as causative agents of infections in humans and animals. The taxonomic status of *Prototheca* has evolved over several decades, and four species are currently assigned to the genus: *Prototheca zopfii*, *Prototheca wickerhamii*, *Prototheca stagnora* and *Prototheca ulmea*. A fifth not generally accepted species was assigned as *Prototheca moriformis* (Arnold & Ahearn, 1972; Krüger, 1894; Pore, 1985, 1986). This species is genetically and biochemically very similar to *P. zopfii*; otherwise, there is a marked heterogeneity between strains of *P. moriformis* (Pore, 1985; Ueno *et al.*, 2003).

In addition, three biotypes or so-called 'variants' of *P. zopfii* have been defined, based on phenotypic characteristics (Roesler *et al.*, 2003). Auxanographical and biochemical investigations of isolates from various habitats revealed that

all bovine mastitis isolates (biotype 2) showed a delayed assimilation of galactose and an increased assimilation of amino acids, compared with isolates of biotype 1 from cattle liquid manure (Blaschke-Hellmessen *et al.*, 1985; Roesler *et al.*, 2003). Isolates from swine farms (biotype 3) were not capable of assimilating glycerol or the majority of the amino acids tested. Furthermore, serological typing by immunoblotting revealed major differences in the pattern of immunogenic structures between the three *P. zopfii* biotypes (Roesler *et al.*, 2003). Comparative investigations by means of Fourier-transformed infra-red spectroscopy also showed distinct differences between variant 3 and the other two variants. However, discrimination of variants was not possible between strains assigned to variant 1 or 2 (Schmalreck *et al.*, 1998).

The present study involved the comparative investigation of the three biotypes by means of sequence analysis of the 18S rRNA gene and determination of cellular fatty acids. The data showed that the three biotypes clearly differ in both the small-subunit (18S) rRNA gene and cellular fatty acid composition. Furthermore, the sequences of members of the current biotype 3 differ markedly from those of the other described *Prototheca* species. Therefore, a novel species,

Abbreviations: ML, maximum-likelihood; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession number for the 18S rRNA gene sequence of *Prototheca blaschkeae* sp. nov. SAG 2064^T is AY973041.

A figure showing aligned target sequences of specific oligonucleotides for *P. zopfii* genotype 1, *P. zopfii* genotype 2 and *P. blaschkeae* sp. nov. is available as supplementary material in IJSEM Online.

Prototheca blaschkeae sp. nov., is proposed for the present biotype 3. In addition, two novel genotypes 1 and 2 are proposed for the current biotypes 1 and 2 of *P. zopfii*.

For this study, *P. zopfii* isolates from bovine mastitis from different herds (RZII-2 and RZII-3), from different cattle liquid manure and cattle barns (RZI-1, RZI-2 and RZI-3), from different piggeries (RZIII-1 and RZIII-2) and from a human case of onychomycosis (RZIII-3^T) were provided by the Institute of Microbiology and Hygiene of the Technical University of Dresden, Dresden, Germany. These strains were originally isolated by R. Blaschke-Hellmessen. In addition, one strain (LZ 5) was isolated from a clinical case of severe mastitis in a lactating cow. More recently, this strain has been used as an ELISA antigen for the serological diagnosis of bovine protothecal mastitis (Roesler *et al.*, 2001). Three of these strains were deposited at the Stammsammlung für Algenkulturen (SAG), University of Göttingen, Göttingen, Germany. Strain RZI-3 was deposited as SAG 2063, LZ 5 as SAG 2021 and RZIII-3^T as SAG 2064^T. In addition, *P. zopfii* strain SAG 263-4^T, which was isolated from a human case of enteropathy, was also used for the comparative investigation. The strains investigated were biochemically assigned to biotype 1 (RZI-1, RZI-2 and SAG 2063), biotype 2 (SAG 263-4^T, SAG 2021, RZII-2 and RZII-3) and biotype 3 (RZIII-1, RZIII-2 and SAG 2064^T) of *P. zopfii* (Blaschke-Hellmessen *et al.*, 1985; Roesler *et al.*, 2003). In addition, two other strains (from the Central Bureau voor Schimmelcultures, Utrecht, The Netherlands) were used for biochemical discrimination: *P. stagnora* CBS 605.66 and *P. wickerhamii* CBS 157.74. Biochemical identification of *Prototheca* species and differentiation into biotypes of *P. zopfii* were performed as described previously (Roesler *et al.*, 2003) by using the microbial identification system BBL Crystal (Becton Dickinson). Two kits were utilized, BBL Crystal Enteric/Nonfermenter and BBL Crystal Gram positive. In total, 50 carbon and nitrogen sources were tested with these two kits for each strain. The test panels were incubated in a humid chamber at 37 °C for 48 h. Biochemical characteristics that discriminated *Prototheca* species are shown in Table 1.

With the aim of achieving intraspecies and interspecies differentiation, the strains were compared by means of their cellular fatty acid pattern, sequence analysis of the highly conserved 18S rRNA genes and by biotype-specific PCR.

The *Prototheca* strains investigated were cultured aerobically on Sabouraud dextrose agar plates for 48 h. Fatty acid methyl esters (FAMES) were extracted from 100 mg biomass of the isolates, as described by Biedermann *et al.* (2004) with minor modifications. The extraction was performed with n-hexane/methanol (1:1, by vol.), with stirring over a 60 min period. Subsequently, the FAMES were separated by GC/MS using an Agilent 6890N gas chromatograph and an Agilent 5973N mass selective detector (Agilent Technologies). MSD Productivity Enhanced ChemStation software (Agilent Technologies) was used to acquire the MS

Table 1. Characteristics that discriminate *Prototheca* species

Strains: 1, *P. stagnora* CBS 605.66; 2, *P. wickerhamii* CBS 157.74; 3, *P. zopfii* genotype 1 SAG 2063; 4, *P. zopfii* genotype 2 SAG 2021; 5, *P. blaschkeae* sp. nov. SAG 2064^T. Nutrient utilization is scored as: +, utilization; –, no utilization; (–), utilization after 96 h. Fatty acids shown are only those that differentiate *P. zopfii* genotypes and *P. blaschkeae* sp. nov. (out of 21 detected). Values given are means plus SEM (in parentheses). NI, Not investigated.

Characteristic	1	2	3	4	5
Nutrient utilization*:					
Galactose ^a	(–)	+	+	(–)	+
Glycerol ^a	+	+	+	+	–
Arginine ^a	+	+	+	+	–
Lysine ^a	(–)	+	–	+	–
Fructose ^b	+	+	+	+	+
Trehalose ^b	–	+	–	–	–
Capsule	+	–	–	–	–
Fatty acid (%):					
C _{16:0}	NI	NI	23.14 (0.26)	23.3 (0.89)	15.58 (0.42)†
C _{16:1}	NI	NI	0.38 (0.02)	0.28 (0.02)	1.45 (0.06)†
C _{17:2} –CP _{17:0}	NI	NI	0.36 (0.09)	0.12 (0.05)	1.26 (0.16)†
C _{18:1}	NI	NI	24.46 (1.18)	29.02 (1.01)	39.94 (1.22)†
C _{20:2}	NI	NI	0.02 (0.02)	0.36 (0.02)†	0.0

*Determined after 48 h using BBL Crystal kits: a, Enteric/NF; b, Gram positive.

†Significantly different ($P < 0.01$) from the other strains investigated.

data. The FAMES were then identified using the NIST 98 mass spectral library (Agilent Technologies). Cultivation followed by analysis was repeated five times. These fatty acid analyses showed that there were clear differences between the current biotypes of *P. zopfii* (Table 1). Essentially, the differences detected were a significantly ($P < 0.01$) increased amount of eicosadienoic acid (C_{20:2}) in biotype 2 compared with biotype 1 and with biotype 3 that does not contain this fatty acid, and significantly increased amounts of palmitic acid (C_{16:0}), palmitoleic acid (C_{16:1}), linoleic acid–cyclopropane complex (C_{17:2}–CP_{17:0}) and oleic acid (C_{18:1}) in biotype 3 compared with biotypes 1 and 2.

Preparation of genomic DNA was carried out as described previously (Roesler *et al.*, 2001). In brief, the algal cells were ultrasonicated and the DNA was obtained using a DNeasy Plant kit (Qiagen). For sequence analysis of the 18S rRNA gene, four overlapping fragments of the entire gene were amplified using the primer pairs Proto18-1f (5'-GATAC-AAGACCGACCGCCTTT-3') and Proto18-1r (5'-AAACC-GTTAGCTTTACTCCC-3'), Proto18-2f (5'-CGCGCAAA-

TTACCCAATCC-3') and Proto18-2r (5'-TGAGACCGG-ACAGGGCAA-3'), Proto18-3f (5'-CCTCTGCAGGACCG-GAGTAA-3') and Proto18-3r (5'-TGTACCGCTCCTAAC-TGTCTA-3'), and Proto18-4f (5'-GACATGGCGAGGATT-GACAGA-3') and Proto18-4r (5'-AGGATGGCTAACCC-ACACGA-3').

Based on results of the alignment of the 18S rRNA gene sequences the following oligonucleotides were used for biotype-specific PCR (Fig. 1): PZGT 1/r (5'-GCCAAGG-CCCCCGAAG-3') for biotype 1, PZGT 2/r (5'-GTCG-GCGGGGCAAAGC-3') for biotype 2 and PZGT 3/r (5'-GTTGGCCCGGCATCGCT-3') for biotype 3. These specific reverse primers were used with the following oligonucleotides that also acted as part of the internal amplification controls: Proto18-4f for primers PZGT 1/r and PZGT 2/r; and PZGT 3-IK/f (5'-CAGGGTTCGATTCCGGAGAG-3') for oligonucleotide PZGT 3/r. The alignment with the target regions of these oligonucleotides is available as Supplementary Fig. S1 in IJSEM Online.

The following oligonucleotides served as internal amplification controls: Proto18-4f and Proto18-4r for biotypes 1 and 2; PZGT 3-IK/f and PZGT 3-IK/R (5'-GAATTACCGCG-GCTGCTGG-3') for biotype 3. PCR was carried out using Mastermix 1.1 × ReddyMix (Abgene) in a Tpersonal thermal cycler (Whatman Biometra). Cycle conditions were 30 s denaturation at 94 °C and 40 s extension at 72 °C. Annealing conditions were chosen based on the G + C content of the corresponding oligonucleotides (58 °C for biotypes 1 and 2; 63 °C for biotype 3). The products were analysed on a 1 % (w/v) agarose gel and purified using a PCR Purification kit (Qiagen). They were then sequenced using an ABI Prism 3100 Avant sequencer (Applied Biosystems) using the dideoxy-chain termination method. The sequences were analysed using DNA Sequencing analysis software v5.2 (Applied Biosystems).

To examine phylogenetic relationships, the sequences were first aligned using CLUSTAL W multiple sequence alignment (Thompson *et al.*, 1994), with the MegAlign module of the LASERGENE software package (DNASTAR). Available reference sequences (GenBank) of *Prototheca* species were included. Phylogenetic analyses were performed using TREECON (Van de Peer & De Wachter, 1994) and TREEFINDER (Jobb *et al.*, 2004). The aligned sequences were subjected to genetic distance calculation using the two-parameter method of Kimura (1980) and to phylogenetic analysis using the neighbour-joining (NJ) method (Saitou & Nei, 1987) with TREECON and the maximum-likelihood (ML) method with TREEFINDER. Bootstrap analyses were performed on 1000 random samples (100 samples for ML) taken from multiple alignments, as described by Felsenstein (1985).

Based on the results of the sequence analysis, the current biotypes of *P. zopfii* should be reclassified as genotypes 1 (biotype 1) and 2 (biotype 2), and as a novel species, *P. blaschkeae* sp. nov. (biotype 3).

Fig. 1. Results of biotype-specific PCRs for *P. zopfii* genotype 1 (a), *P. zopfii* genotype 2 (b) and *P. blaschkeae* sp. nov. (c) showing the specific products (*P. zopfii* genotype 1, 150 bp; *P. zopfii* genotype 2, 165 bp; *P. blaschkeae*, 126 bp) and the internal amplification controls (*P. zopfii* genotypes 1 and 2, 450 bp; *P. blaschkeae*, 213 bp). Lanes: M, Marker; 1, *P. zopfii* genotype 1 RZ1-1; 2, *P. zopfii* genotype 1 RZ1-2; 3, *P. zopfii* genotype 1 SAG 2063; 4, *P. zopfii* genotype 2 SAG 2021; 5, *P. zopfii* genotype 2 RZII-2; 6, *P. zopfii* genotype 2 RZII-3; 7, *P. blaschkeae* sp. nov. RZIII-1; 8, *P. blaschkeae* sp. nov. RZIII-2; 9, *P. blaschkeae* sp. nov. SAG 2064^T.

Comparison of the partial sequences of the 18S rRNA genes revealed clear differences between the strains of the three current biotypes and all strains of each biotype were conspecific. The sequences of biotype 3 (strains RZIII-1, RZIII-2 and SAG 2064^T; GenBank accession no. AY973041) clearly differed from those of biotypes 1 and 2 and had a sequence similarity of 98.1 % in the investigated 1442 bp of the 18S

rRNA gene. The sequences of biotype 2 (strains RZII-2, RZII-3, SAG 263-4^T and SAG 2021; AY940456) differed in some nucleotides from those of biotype 1 (strains RZI-1, RZI-2 and SAG 2063; AY973040) and had a sequence

similarity of 99.4% with biotype 1. Phylogenetic analysis with the NJ method showed that all biotypes were most closely related to the other strains of *P. zopfii* and *P. moriformis* (Fig. 2a). Although the strains originated from

Fig. 2. Phylogenetic relationships of *P. zopfii*, *P. blaschkeae* sp. nov. and other recognized *Prototheca* species, based on 18S rRNA gene sequences. The trees were constructed using the NJ (a) and ML (b) methods. Numbers at nodes are percentage bootstrap frequencies derived from 1000 (NJ) or 100 (ML) replicates. GenBank accession numbers are shown in parentheses. Bar, genetic distance between the strains analysed.

epidemiologically different sources, the investigated strains of each biotype are clearly conspecific and form three distinct clades. Thereby, the strains of biotype 3 show the greatest phylogenetic distance from the other known *P. zopfii* sequences, including the sequences of *P. zopfii* biotypes 1 and 2. However, the investigated strains of biotypes 1 and 2 show a clearly smaller distance from strains of the *P. zopfii* clade. Thereby, biotype 1 differs more clearly than biotype 2 (Fig. 2a). The results obtained using the ML method were substantially the same (Fig. 2b).

The findings of previous serological, biochemical and morphological studies (Blaschke-Hellmessen *et al.*, 1987, 1985; Roesler *et al.*, 2003; Schmalreck *et al.*, 1998) were confirmed by our biochemical and genetic results. One consequence of these results is the need to divide the species *P. zopfii* into at least two genotypes and the proposal of a novel *Prototheca* species.

One unanswered question is the assessment of the two established genotypes as two novel subspecies of *P. zopfii*. Strains of genotype 1 show marked biochemical and antigenic differences from other *P. zopfii* strains and furthermore form a well-supported phylogenetic clade. Because of the difficulty of biochemical identification, a possible subspecies name for the current biotype 1 could be *Prototheca zopfii* subsp. *occulta*. The subspecies status of genotype 2 of *P. zopfii* is strongly supported by typical disease-associated occurrence of the current biotype 2 in bovine mastitis, which has not yet been reported for the other biotypes (Schuster & Blaschke-Hellmessen, 1983). Therefore, a possible name for this subspecies could be *Prototheca zopfii* subsp. *bovima-titogenes*. Furthermore, this genotype has obvious differences in antigenic pattern as well as in the pattern of cellular fatty acids. In contrast, based on 18S rRNA gene sequences, the strains of this genotype are most closely related to other currently recognized *P. zopfii* strains, including *P. moriformis*. However, all investigated strains of biotype 2 are genetically conspecific and show marked differences from the other *P. zopfii* strains, which all show slight differences at the 18S rRNA gene level. In this context, it should be discussed whether the current genetically analysed strains of *P. zopfii* (e.g. *P. zopfii* var. *hydrocarborea*) and *P. moriformis* also belong to different subspecies of *P. zopfii*. Our results clearly indicate that *P. moriformis* should be assigned to *P. zopfii* and does not represent an independent and valid species, supporting the results of Ueno *et al.* (2003). However, the known strains of *P. moriformis* have a strong heterogeneity in their biochemical characteristics, suggesting different taxonomic clusters within these strains.

Therefore, biotyping of the currently genetically analysed *P. zopfii* and *P. moriformis* strains is necessary and additional comparative sequence analyses of other genes, e.g. the large-subunit rRNA gene, are required to clarify the taxonomic status of these potential subspecies. Such studies are also necessary to elucidate the taxonomic position of *P. wickerhamii*. *P. wickerhamii* is more closely related to the genus *Chlorella* than to the genus *Prototheca* and should, therefore,

be assigned to the genus *Chlorella*. This supports the phylogenetic relationships within the genus *Prototheca* reported by Ueno *et al.* (2003).

Proposed type strains for the described two genotypes of *P. zopfii* are SAG 2063 (GenBank accession no. AY973040) for *P. zopfii* genotype 1 and SAG 2021 (AY940456) for *P. zopfii* genotype 2, which have both been deposited in the Stammsammlung für Algenkulturen.

Finally, morphological results from the Fourier-transformed infra-red spectroscopy that showed distinct differences between biotype 3 and the other two biotypes (Schmalreck *et al.*, 1998), the pattern of cellular fatty acids and the results of the sequence analysis indicate that the investigated isolates of the current biotype 3 of *P. zopfii* belong to a novel *Prototheca* species, *Prototheca blaschkeae* sp. nov.

Latin diagnosis of *Prototheca blaschkeae* Roesler, Möller, Hensel, Baumann et Truyen sp. nov.

In agar Sabouraudii post 4 dies ad 37 °C, candida vel crenea, glabra, margo glabro vel undulato (2 mm). Textura molis. Sporangiosporae ovoideae aut globosae (5–7.5 µm) et sporangiae globosae (15 µm). Ad 28 °C et 37 °C semper crescit. Glucosum, galactosum et fructosum assimilantur at non glycerolum, sorbitolum, arabinosum, lactosum, mannosum, melobiosum, rhamnosum, sucrosus et trehalosum. Phenylalaninum et prolinum assimilantur at non lysinum, valinum et tetrazolium.

Typus RZIII-3^T (= SAG 2064^T), ex onychomycosi hominis, Dresden, Germania, R. Blaschke-Hellmessen isolatus est. In collectione Stammsammlung für Algenkulturen, Universitas Göttingen, Göttingen, Germania, SAG 2064^T deposita est.

Description of *Prototheca blaschkeae* Roesler, Möller, Hensel, Baumann & Truyen sp. nov.

Prototheca blaschkeae (bla.sch'ke.ae. N.L. gen. n. *blaschkeae* in honour of Professor Renate Blaschke-Hellmessen, Dresden, Germany, who first described the occurrence of different 'variants' of *P. zopfii*).

After 4 days incubation on Sabouraud dextrose agar at 37 °C, colonies are white to cream, smooth, convex and have a mean diameter of 2 mm. Texture of colonies is soft and viscous. Form small, ovoid to globose (5–7.5 µm) sporangiospores in sporangias, with a mean diameter of 15 µm (Fig. 3). Good growth occurs at 28 and 37 °C. Glucose, galactose and fructose are assimilated, but not glycerol, sorbitol, arabinose, lactose, mannose, melobiose, rhamnose, sucrose or trehalose. Phenylalanine and proline are assimilated, but not lysine, valine or tetrazolium.

The type strain, RZIII-3^T, was isolated from a human case of onychomycosis in Dresden, Germany, by R. Blaschke-Hellmessen and has been deposited in the Stammsammlung

Fig. 3. Light micrographs of cells of *P. zopfii* genotype 1 SAG 2063 (a), *P. zopfii* genotype 2 SAG 2021 (b) and *P. blaschkeae* sp. nov. SAG 2064^T (c) stained with lactophenol cotton blue. Arrows indicate sporangia (1) and released sporangiospores (2). The cultures were photographed after an incubation period of 48 h at 37 °C on Sabouraud dextrose agar plates. *P. zopfii* genotype 2 SAG 2021 forms substantially smaller daughter cells (sporangiospores), with a diameter of about 5 µm. In contrast, sporangiospores of *P. zopfii* genotype 1 SAG 2063 and *P. blaschkeae* SAG 2064^T are approximately 10 µm in size. Bars, 15 µm.

für Algenkulturen, University of Göttingen, Göttingen, Germany, as SAG 2064^T. Reference strains are RZIII-1 and RZIII-2.

Micrographs of *P. blaschkeae* SAG 2064^T, *P. zopfii* genotype 1 SAG 2063 and *P. zopfii* genotype 2 SAG 2021 are shown in Fig. 3.

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