Emended phenotypic characterization of *Prototheca zopfii*: a proposal for three biotypes and standards for their identification

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A representative selection of *Prototheca zopfii* strains isolated from different environmental habitats or clinical cases was characterized in a polyphasic approach in order to assess their intraspecies taxonomic position. Recently, the recognition of distinct phenotypic clusters has been reported as the assignment of 'variants'. In the present study, 11 strains were compared by a number of phenotypic and genetic criteria, including growth characteristics, biochemical reactions and serotyping results. Based on emended standards for biotype identification, *P. zopfii* strains showed auxanographic differences and distinct assimilation patterns with respect to utilization of amino acids and glycerol. Serotyping by means of immunoblotting revealed that all isolates of variant II obtained from clinical cases, i.e. isolates from bovine mastitis or from human enteropathia, showed specific antigen patterns. They were found to be different from strains assigned to the other two variants with respect to their immunogenic antigens. Furthermore, comparison of partial 18S rDNA sequences confirmed distinct differences between the former variants. Based on these results, it is proposed that *P. zopfii* merits classification as a species comprising three biotypes.

Colourless algae of the genus *Prototheca*, family *Chlorellaceae*, are the only known plants that are causative agents of infections in humans and animals. The taxonomic status of *Prototheca* has evolved in recent decades, and four species are currently assigned to the genus: *Prototheca zopfii*, *Prototheca wickerhamii*, *Prototheca stagnora* and *Prototheca ulmea*. A fifth species was assigned to *Prototheca moriformis* (Arnold & Ahearn, 1972; Pore, 1985, 1986).

Numerous reports have indicated a pathogenic potential for *P. zopfii* and *P. wickerhamii*. *P. wickerhamii* is predominantly isolated from clinical cases of human infections (Bianchi *et al.*, 2000; Matsuda & Matsumoto, 1992). *P. zopfii* causes infections in animals, particularly of dairy cows or dogs (Blogg & Sykes, 1995; Ginel *et al.*, 1997; Schultze *et al.*, 1998). Worldwide, *P. zopfii* has been identified as inducing a therapy-resistant inflammation of the mammary gland in dairy cows that may lead to severe economic losses in an infected herd. Isolates assigned to variant II were reported to be the predominant infectious agents (Costa *et al.*, 1998; Janosi *et al.*, 2001; Jensen *et al.*, 1998). Therefore, the correct identification of *P. zopfii* isolates is important in clinical microbiology laboratories.

At present, accurate identification is based on phenotypic characteristics. Several phenotypic studies support the recognition of distinct clusters and they have been named as 'variants', as suggested by Blaschke-Hellmessen *et al.* (1985). *P. zopfii* includes strains isolated from various animal hosts that are distinct from related strains of environmental origin. *P. zopfii* has consistently been divided into three variants by different reports. Auxanographic investigations of different isolates revealed that all bovine mastitis isolates showed delayed assimilation of galactose. Thus, the bovine mastitis isolates were assigned to variant II. Isolates from swine farms (variant III) were not able to assimilate glycerol (Blaschke-Hellmessen *et al.*, 1985). Comparative investigations by means of Fourier-transformed infra-red spectroscopy showed distinct differences between variant III and the other two variants. However, discrimination was not possible between strains assigned to variants I or II (Schmalreck *et al.*, 1998). Furthermore, serological typing on the basis of counter immunoelectrophoresis yielded inconsistent results. While whole-cell antigen of variant II raised several precipitation lines, antigens prepared from the other two variants showed significantly fewer precipitation lines (Blaschke-Hellmessen *et al.*, 1987).

Accurate identification is impaired due to variable expression of certain traits such as typing by counter immunoelectrophoresis and because of frequent ambiguity in the

interpretation of such reactions. The aim of this study was to perform an extensive phenotypic analysis in order to provide emended standards for *P. zopfii* identification. Our results reveal that *P. zopfii* comprises three different phenotypic clusters that correspond to three distinct biotypes. On the basis of these data, we propose the dissection of the species *P. zopfii* into three biotypes.

The type strain of *P. zopfii*, SAG 263-4^T, originally isolated from a human case of enteropathia, and strain SAG 2021, isolated from a clinical case of a severe acute mastitis in a lactating cow, were obtained from the Stammsammlung für Algenkulturen (SAG), University of Göttingen, Germany. More recently, SAG 2021 was used as ELISA antigen for serological diagnosis of bovine protothecal mastitis (Roesler *et al.*, 2001). In addition, *P. zopfii* isolates from bovine mastitis (RZII-1, RZII-2, RZII-3), cattle liquid manure and cattle barns (RZI-1, RZI-2, RZI-3), piggeries (RZIII-1, RZIII-2) and a human case of onychomycosis (RZIII-3) were provided by the Institute of Microbiology and Hygiene of the Technical University of Dresden, Germany. These strains have been assigned to variants I (RZI-1, RZI-2, RZI-3), II (RZII-1, RZII-2, RZII-3) and III (RZIII-1, RZIII-2, RZIII-3) (Blaschke-Hellmessen *et al.*, 1985).

Classification of the different strains into biotypes of *P. zopfii* was performed auxanographically by means of assimilation of glucose, glycerol and galactose on *Prototheca* isolation medium (Pore, 1973). Plates were incubated for 48 h at 37 °C as recommended for variant assignment (Blaschke-Hellmessen *et al.*, 1985).

In order to detect further biochemical differences, *P. zopfii* strains were also investigated by a microbial identification system (BBL Crystal; Becton Dickinson). Two kits of this identification system were utilized, BBL Crystal Enteric/Nonfermenter and BBL Crystal Gram-positive. In total, 50 different carbon and nitrogen sources were checked for each strain with these two kits. The test panels were incubated in a humid chamber at 37 °C for 48 h.

In order to clarify the assignment of different strains to biotypes of *P. zopfii*, algal strains were investigated by Western blotting. Therefore, specific hyperimmune sera directed against *P. zopfii* were developed in rabbits. A total of 10⁷ cells (ml PBS⁻¹) of either strain RZI-3 (biotype I), SAG 2021 (biotype II) or RZIII-3 (biotype III) were emulsified with an equal volume of Freund's incomplete adjuvant (Sigma); 1 ml was administered intradermally to New Zealand White rabbits with an approximate body mass of 2.5–3.5 kg. Starting at 3 weeks post-priming, the rabbits were boosted intravenously three times biweekly with 10⁷ viable cells of the homologous strain. At 7 days after the last application (10 weeks after priming), the rabbits were bled and sera were collected.

Prototheca cells were resuspended in distilled water, broken by repeated freezing and thawing cycles in liquid nitrogen, ultrasonicated and heated (100 °C, 5 min). Protein fractions

of each preparation were separated on 10 % denaturing SDS-PAGE gels. Proteins were transferred to a PVDF membrane for immunoblotting. In order to minimize unspecific background reactions, a synthetic blocking reagent was used (Roti-Block; Carl Roth). Peroxidase-conjugated anti-IgG antibodies derived from goats (Bethyl Laboratories) were used as second antibodies to detect serum antibodies of immunized rabbits. Detection was performed by chemiluminescence using ECL kits (Amersham Pharmacia Biotech).

In addition to biochemical and serological analysis, strains SAG 2021, SAG 263-4^T (both variant II), RZI-3 (variant I) and RZIII-3 (variant III) were compared by partial 18S rDNA sequence analysis. Genomic DNA was prepared as described recently (Roesler *et al.*, 2001). For amplification of the 18S rDNA, primers wicker-18f (5'-AACCTGGT-TGATCCTGCCAGT-3') and wicker-18r (5'-TGATCCT-TCTGCAGGTTACC-3') were used. PCR amplification was carried out in a Perkin-Elmer 2400 thermal cycler. The PCR fragment was sequenced directly using the internal primer wicker-18S-fseq1 (5'-TGCCAGTAGTCATATGCT-TGT-3') and further sequence-derived oligonucleotides. Nucleotide sequence determination was carried out with a LI-COR DNA sequencer model 4000 by the dideoxy chain-termination method. The sequence was analysed using the Wisconsin Package GCG software version 8.1.

Based on the results presented here, members of the species *P. zopfii* appear to possess diverse chemotaxonomic features with respect to their antigenic and biochemical properties. All isolates of the former variant strains proposed for *P. zopfii* were found to be consistent with their assignment to the species *P. zopfii*. However, the phenotypic patterns did not correspond completely to hitherto established variants of this species. Thus, based on phenotypic evidence, we consider that *P. zopfii* isolates should be assigned to three biotypes.

Our auxanographic investigations showed that a strong assimilation activity of galactose and glycerol within 48 h indicated *P. zopfii* biotype I. *P. zopfii* biotype II did not show assimilation of galactose within 48 h, whereas *P. zopfii* biotype III was not able to utilize glycerol at all. Our results confirm distinct differences between isolates of *P. zopfii* that originated from different habitats (Table 1). When compared with biotype II, biotype I showed substantially stronger assimilation of galactose. However, time-dependent galactose assimilation must be evaluated critically and may be determined by medium or strain origin. Isolates of variant III were characterized by the fact that they could not utilize glycerol, while isolates of variants I and II did.

Examination of assimilation of 50 different carbon and nitrogen sources showed definite differences between the three biotypes of *P. zopfii*. *P. zopfii* strains isolated from cow barns are said to be biotype I (or variant) if, among other traits, they utilize galactose and glycerol, and biotype

Table 1. Characteristics for differentiation of biotypes I, II and III in *P. zopfii*

Auxanographic reactions are scored as: –, no growth; (+), doubtful; +, weak; ++, moderate; +++, strong. Strong growth of all strains was observed with glucose after 48 h. Nutrient utilization was determined after 48 h using BBL Crystal kits and is scored as: +, utilization; –, no utilization.

Characteristic	Biotype I			Biotype II					Biotype III		
	RZI-1	RZI-2	RZI-3	SAG 263-4 ^T	SAG 2021	RZII-1	RZII-2	RZII-3	RZIII-1	RZIII-2	RZIII-3
Source	Cow barn	Cow barn	Cow barn	Human enteropathia	Bovine mastitis	Bovine mastitis	Bovine mastitis	Bovine mastitis	Piggery	Piggery	Human onychomycosis
Auxanographic reactions:											
Glycerol (48 h)	+++	+++	+++	+++	+++	+++	+++	+++	–	–	–
Galactose (24 h)	(+)	+	+	–	–	–	–	–	(+)	(+)	(+)
Galactose (48 h)	++	++	++	(+)	–	–	(+)	(+)	+	++	++
Galactose (72 h)	+++	+++	+++	+	+	(+)	+	(+)	++	++	+++
Nutrient utilization:											
Glycerol	+	+	+	+	+	+	+	+	–	–	–
Galactose	+	+	+	–	–	–	–	–	+	+	+
Glucoside	+	+	–	–	+	+	+	+	–	–	–
Inositol	–	–	+	–	–	–	–	–	–	–	–
Tetrazolium	+	–	+	+	+	+	–	–	–	–	–
Tryptophan	–	+	–	–	+	+	+	–	–	+	–
Valine	–	+	+	–	+	+	+	+	–	+	–
Arginine	+	+	+	+	+	+	+	+	–	–	–
Lysine	–	–	–	+	+	+	+	+	–	–	–

II (or variant) if they utilize glycerol but not galactose within 48 h. The three strains that clustered with the *P. zopfii* type strain SAG 263-4^T (originally isolated from human enteropathia) and strain SAG 2021 (originally isolated from bovine mastitis) were also galactose-negative and compatible with biotype II, as is the type strain, and were also similar to the published description of *P. zopfii* variant II (Blaschke-Hellmessen *et al.*, 1985). Biotype III strains are distinguished by the ability of the latter group to utilize glucose but not glycerol. Table 1 gives characteristics useful in the differentiation of the biotypes of *P. zopfii*. Major differences in the pattern of utilization of amino acids were found among the biotypes. Strains of biotype I were not able to utilize lysine, whereas all isolates of biotype II tested were found to be positive. Strains of biotype III differed most clearly from the utilization patterns typical for the two other biotypes. In addition to the inability to assimilate glycerol, the inability to utilize most amino acids allowed the differentiation of biotype III from the other biotypes (Table 1).

The immunogenic properties of the different biotypes are shown in Fig. 1. Major differences between the three biotypes of *P. zopfii* are detectable in the pattern of immunogenic structures. Isolates of biotype III showed a specific antigen at 37 kDa, while biotype II strains yielded a specific antigen at 51 kDa. These biotype-specific antigens were detected by all hyperimmune sera tested. It can be assumed that these proteins represent cross-reacting but biotype-specific antigens. On the basis of these results, the existence of at least three different serotypes of *P. zopfii* can be postulated. However, as can be seen in Fig. 1, it is obvious that small serological differences exist among strains of biotype II.

Comparison of partial 18S rDNA sequences showed clear differences between two clinical isolates of biotype II (SAG 263-4^T and SAG 2021) and strains of biotypes I (RZI-3) and III (RZIII-3). The strains of biotype II showed 99.4 % sequence similarity to the other strains examined. Biotype I differed in some nucleotides from the two other variants and, hence, it showed 99.8 % sequence similarity.

The results of earlier serological, auxanographic and morphological studies (Blaschke-Hellmessen *et al.*, 1985, 1987; Schmalreck *et al.*, 1998) were confirmed by our findings. The definite biochemical and serological differences between the clusters of isolates support the suggestion to establish at least three distinct biotypes within the algal species *P. zopfii*. This hypothesis is strongly supported by the typically illness-associated occurrence of biotype II, which has not been reported for the other biotypes (Schuster & Blaschke-Hellmessen, 1983). Among the authors of this manuscript, there have been many discussions concerning how to treat the heterogeneity within the species *P. zopfii*. Our genetic analyses even suggest the existence of different subspecies. One possibility would have been to dissect the species *P. zopfii* into three subspecies, but this is premature.

Fig. 1. Serological comparison of *P. zopfii* biotypes. Rabbit hyperimmune sera raised against strains RZI-3 (a; biotype I), SAG 263-4^T (b; biotype II) and RZIII-3 (c; biotype III) were used as primary antibodies in immunoblots. Lanes: 1, SAG 263-4^T (biotype II); 2, SAG 2021 (II); 3, RZI-1 (I); 4, RZI-3 (I); 5, RZII-1 (II); 6, RZII-3 (II); 7, RZIII-1 (III); 8, RZIII-3 (III). Specific antigens of biotype II at 51 kDa (#) and of biotype III at 37 kDa (*) are indicated. A common antigen of all biotypes at 30 kDa is indicated by §.

While we wish to recognize the heterogeneity within the species, we felt that it would not be appropriate to give these variant clusters subspecies status, but to elect to

divide them on a formal basis into three different biovars. The recognition of biovars within the species *P. zopfii* has the advantage that one can differentiate the three groups without introducing nomenclatural changes. The results we have presented and the conclusions we have drawn indicate that a closer study of a larger number of isolates may provide an interesting insight into the diversity within this species. In particular, in order to clarify the epidemiological role of biotype II in protothecosis in man and in dairy cows, more clinical isolates of *P. zopfii* should be investigated.

In summary, this study proves the existence of evident biochemical and serological differences within the algal species *P. zopfii*. Based on phenotypic evidence, it is proposed that *P. zopfii* isolates should be assigned to three biotypes. Therefore, in future, variants should be termed biotypes I, II and III. A phylogenetic analysis of the complete 18S rRNA gene sequences should be performed in future in order to reveal the relationships of these taxa.

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