Biotechnology, physiology and genetics of the yeast Pichia anomala

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MINI REVIEW

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Abstract

The ascomycetous yeast Pichia anomala is frequently associated with food and feed products, either as a production organism or as a spoilage yeast. It belongs to the nonSaccharomyces wine yeasts and contributes to the wine aroma by the production of volatile compounds. The ability to grow in preserved food and feed environments is due to its capacity to grow under low pH, high osmotic pressure and low oxygen tension. A new application of P. anomala is its use as a biocontrol agent, which is based on the potential to inhibit a variety of moulds in different environments. Although classified as a biosafety class-1 organism, cases of P. anomala infections have been reported in immunocompromised patients. On the other hand, P. anomala killer toxins have a potential as antimicrobial agents. The yeast can use a broad range of nitrogen and phosphor sources, which makes it a potential agent to decrease environmental pollution by organic residues from agriculture. However, present knowledge of the physiological basis of its performance is limited. Recently, the first studies have been published dealing with the global regulation of the metabolism of P. anomala under different conditions of oxygenation.

Introduction

The yeast Pichia anomala (E.C. Hansen) Kurtzman has been isolated from a variety of habitats, both man-made and natural (Kurtzman, 1998). It is frequently found in fermented drinks and foods where it plays a role in the spontaneous fermentation (Masoud et al., 2004; Sujaya et al., 2004), and belongs to the nonSaccharomyces wine yeasts (Rojas et al., 2003).

Pichia anomala can grow under extreme environmental stress conditions, such as low and high pH, low water activity, high osmotic pressure and anaerobic conditions (Fredlund et al., 2002). Due to these characteristics, this yeast can be a spoilage organism, for instance in high-sugar food products (Tokuoka et al., 1985; Lanciotti et al., 1998) or silage (Jonsson & Pahlow, 1984). Pichia anomala has also been found in clinical isolates and is regarded as an opportunistic pathogen in severely immunocompromised hosts (Hazen, 1995). Although able to grow over a broad pH range and at high osmotic pressure, P. anomala is not particularly tolerant against ethanol and acetate (Kalathenos et al., 1995; Fredlund et al., 2002).

The yeast also inhibits a variety of fungi in several different habitats (Passoth & Schnürer, 2003) and is a potential candidate for biocontrol of moulds on grapevine and during postharvest storage of apples and airtight-stored grain (Jijakli & Lepoivre, 1998; Petersson & Schnürer, 1998; Masih et al., 2000; Druvefors et al., 2002).

The physiology and genetics of this yeast are poorly characterized. The first reports on a general physiological and genetic characterization, as well as molecular genetic manipulation of P. anomala, have recently been published (Grevesse et al., 2003; Naumov et al., 2001; Fredlund et al., 2004a). The aim of this review is to summarize the current knowledge about this yeast, both from the applied and fundamental points of view.

Taxonomy

Pichia anomala (anamorph Candida pelliculosa Redaelli) is a heterothallic, ascomycetous yeast, forming one to four hat-shaped ascospores (Wickerham & Burton, 1954; Hansen, 1904). It was described for the first time by Hansen (1894) as
Saccharomyces anomalous. Hansen later transferred S. anomalous Hansen together with Saccharomyces saturnus Klöcker to the new yeast genus Willia (Hansen, 1904). However, Sydow & Sydow (1919) transferred all species of the genus Willia to a new genus Hansenula, as the name Willia had been pre-empted for a genus of mosses. The genera Hansenula and Pichia were originally separated on the ability to assimilate nitrate as the only nitrogen source. However, this difference was not regarded to be sufficient to justify the separation into two genera and the species of Hansenula had hat-shaped ascospores were therefore transferred to the genus Pichia (Kurtzman, 1984). The reclassification has now been widely accepted, yet many reports still refer to Hansenula anomala and some argue for reinstating the genus Hansenula (Yamada et al., 1994; Naumov et al., 2001). Those arguments are supported by a phylogenetic study of 500 ascomycete yeasts, where most of the members of the former genus Hansenula (including P. anomala) form a separate clade (Kurtzman & Robnett, 1998).

Genetics

Mating, life cycle, chromosome numbers and strains

Only a few reports exist about the genetics of Pichia anomala. The ploidy level of natural isolates is diploid (Naumov et al., 2001), and haploid heterothallic tester strains for the determination of mating type have been defined, with CBS 1982 referred to as Mat− and CBS 1984 as Mat+ (Wickerham & Burton, 1954; Naumov et al., 2001). Several strains of the anamorph Candida pelliculosa were demonstrated to be haploid and able to mate with one mating type of P. anomala (Wickerham & Burton, 1954). Thus, the inability of the imperfect form to mate and form sexual spores was due to mating incompatibility. Naumov et al. (2001) showed that the ability to mate and sporulate varied between different strains of P. anomala. This was suggested to be due to different mating type capacities. Sexual hybridization between poorly sporulating strains was achieved by mating on selective medium. However, segregation analysis of the mating products suggested tetraploid zygotes. Crossing spores of well-sporulating strains finally generated three strains suitable for genetic analysis. These strains were able to form diploid hybrids with regular meiotic segregation (Naumov et al., 2001). Chromosome numbers as determined by pulsed-field gel electrophoresis were in the range of 9–12, with a size range from 850 to 3500 kb (Naumov et al., 2001). Similar to other species (e.g. Scherer & Magee, 1990; Passoth et al., 1992; Bai et al., 2000), P. anomala strains show a considerable chromosome length polymorphism (Aragao et al., 2001; Naumov et al., 2001; Zagorc et al., 2001; Reccek et al., 2002).

Several P. anomala genes have been cloned and analyzed. This includes genes that can complement auxotrophic mutations, such as URA3 (J.A. Perez et al., unpublished: EMBL accession number Y09221), (Ogata et al., 1992), LEU2 (De la Rosa et al., 2001) and TRP1 (Friel et al., 2003), genes involved in the nitrate assimilation (Garcia-Lugo et al., 2000), an invertase gene (Perez et al., 1996, 2001), SEC61, which is involved in the secretory pathway (Ruiz et al., 2001, 2003), the gene for the alternative oxidase (Sakajo et al., 1999), and the PaEXG2 gene, encoding an exoglucanase that was suggested to be involved in the antifungal activity of P. anomala (Grevesse et al., 2003).

Genetic manipulation

Ogata et al. (1995) were the first to describe a transformation system based on a haploid ura3 strain derived from an isolate from wastewater treatment. The mutant strain was isolated using chemical mutagenesis and subsequent 5-FOA selection. The auxotrophy was complemented by the homologous P. anomala URA3 gene. This gene could also complement a ura3 mutation in Saccharomyces cerevisiae (Ogata et al., 1992). Interestingly, the described sequence of the gene differed from the URA3 gene isolated from another P. anomala strain (EMBL accession number Y09221) by 10 amino acids in the derived protein sequence. This difference might reflect a considerable intraspecific variance of P. anomala. Another transformation system has been developed by Grevesse et al. (2003), also based on the complementation of a ura3 mutation. The vector was used for targeted gene disruption and, similar to other nonconventional yeasts (Klimer & Schäfer, 2004), homologous integration occurred to only 4%.

Genetics of the killer phenotype

Killer activity has been shown in many P. anomala isolates (e.g. Fredlund et al., 2002; Yap et al., 2000). Despite considerable investigations on the killer phenomenon in P. anomala, no genes have been cloned and there is no explicit evidence about the location of the killer genes (Marquina et al., 2002; Passoth & Schnürer, 2003). Because the killer phenotype could not be cured by the application of cycloheximide, elevated temperature, ethidium bromide or acridine orange, it was suggested that the killer genes are chromosomally located (Young & Yagi, 1978; Kagiyma et al., 1988; Vustin et al., 1989). The toxins were considerably different with respect to their activity spectrum, molecular characteristics, molecular weight [toxins of 3–85 kDa and even more than 300 kDa have been described (Kagiyma et al., 1988; Sawant et al., 1989; Vustin et al., 1989; Guyard et al., 1999; Comitini et al., 2004], amino-acid composition, optimum pH and temperature, degree of glycosylation and isoelectric point.
One extremely thermostable killer toxin maintained activity after 5 h incubation at 60 °C, and disappeared only after incubation at 100 °C for 25 min (Vustin et al., 1989).

**Physiology**

**Production of low-molecular metabolites**

Glycerol and arabinitol accumulated in *Pichia anomala* during growth in high-salt environments and on high-sugar substrates (Bellinger & Larher, 1988; Tokuoka et al., 1992). This suggests that both compounds act as compatible solutes and are produced by the cell to cope with the osmotic stress. However, their production pattern differed, with glycerol produced at the beginning of the growth phase and arabinitol towards the end, indicating a different metabolic function. The production of glycerol during osmotic stress has been well documented in fungi (Blomberg & Adler, 1992). Arabinitol is produced by many yeast species, including *Debaryomyces Hansenii*, *Zygosaccharomyces rouxii*, *Zygosaccharomyces bidspur*, *Candida sake* and *Pichia sorbitola*, but not by *Saccharomyces cerevisiae* (Bellinger & Larher, 1988; Van Zyl & Prior, 1990; Tokuoka et al., 1992; Van Eck et al., 1993; Abadias et al., 2000).

In addition to osmotic stress, oxygen supply also influenced the production of both sugar alcohols. Oxygen limitation caused glycerol accumulation inside the cells and in the growth medium (Fredlund et al., 2004b). The flux towards glycerol was twofold higher under oxygen limitation than under aerobic conditions (Fig. 1) (Fredlund et al., 2004a). Arabinitol also accumulated in oxygen-limited cultures, but later than glycerol and mainly during the stationary phase. Similar to glycerol, accumulation of arabinitol was higher in cultures with more severe oxygen limitation. Glycerol is produced under oxygen limitation to reoxidize the redox equivalents produced during amino-acid synthesis (Gancedo & Serrano, 1989). Arabinitol has not yet been reported to be involved in the redox metabolism during fermentative growth. However, as 1 M of NADH is reoxidized during the synthesis of 1 M of arabinitol, it is possible that arabinitol formation also plays a role in the redox balance of the cells.

The nonreducing disaccharide trehalose is one of the major carbon storage compounds in *S. cerevisiae* (François & Parrou, 2001), but it is also regarded as playing a role in stress protection and regulation of the glycolytic flux in this yeast (Thevelein & Hohmann, 1995). In *P. anomala*, maximum accumulation of trehalose has been observed during growth under severe oxygen limitation (Fredlund et al., 2004b). During these conditions, trehalose might be involved in the general stress response towards the increasing ethanol concentration, or as a response to hypoxic stress, as suggested for mammalian and *Drosophila* cells (Chen & Haddad, 2004). It might also be involved in the up-regulation of the glycolytic flux under oxygen limitation (Fredlund et al., 2004a). No trehalose accumulation was found under controlled aerobic cultivation of *P. anomala* (A. Broberg and S. Häkansson, unpublished results).

*Pichia anomala* produced ethanol in oxygen-limited cultures, while in completely aerobic cultures only little ethanol was produced. Acetate was produced during respiratory and respirofermentative growth, but not under severe oxygen limitation (Fredlund et al., 2004a, b). *Pichia anomala* produces a spectrum of small volatile compounds, the major one being ethyl acetate, but other compounds have also been described, such as ethyl propanoate, phenyl ethanol and 2-phenylethyl acetate (Westall, 1999). The metabolic pathways that are responsible for ester synthesis in *P. anomala* differ from those of *S. cerevisiae*. In *S. cerevisiae*, ethyl acetate is mainly produced from acetyl-coenzyme A and ethanol, by the reaction of the enzyme alcohol acetyl transferase (Yoshioka & Hashimoto, 1981). In contrast, *P. anomala* preferentially synthesizes the ester from acetate and ethanol via a reversed esterase reaction (Yoshioka & Hashimoto, 1981; Rojas et al., 2002).

Ethyl acetate was produced by *P. anomala* during growth on glucose and under different aeration conditions. Increased oxygenation reduced the production of ethyl acetate (Gray, 1949; Tabachnick & Joslyn, 1953; Fredlund et al., 2004a). When the culture was shifted from aerobic to oxygen-limited cultivation, the specific production rate increased more than 10-fold (Fig. 2) (Fredlund et al., 2004a). In contrast, Rojas et al. (2001) reported less ethyl acetate production during oxygen-limited conditions as compared with aerated conditions. However, because in these experiments no specific values were given and because the study was not performed under controlled culture conditions, it is not possible to compare directly the different values obtained. Ethyl acetate production requires access to small amounts of oxygen and no ethyl acetate was found in anaerobic cultures (Davies et al., 1951; Tabachnick & Joslyn, 1953). Ethyl acetate has been shown to have an antifungal effect and might contribute to the biocontrol activity of *P. anomala* during airtight storage of grain (Fig. 3) (Fredlund et al., 2004c; Druvefors et al., 2005). Considerable production of the ester has been demonstrated in *P. anomala* growing on grain in test tubes, simulating biocontrol in silos with airtight-stored grain (Fredlund et al., 2004c; Druvefors et al., 2005).

**Regulation of respiration and fermentation at different oxygen supply and on different carbon sources**

*Pichia anomala* is a respiratory or Crabtree-negative yeast, i.e. it produces only marginal amounts of ethanol during aerobic growth on a fermentable carbon source (DeDeken,
Growth of the yeast was inhibited by lack of oxygen, as indicated by linear instead of exponential growth under oxygen limitation (Fredlund et al., 2004b). Under aerobic conditions, only small amounts of ethanol or glycerol were produced, indicating a preferentially respiratory metabolism. A glucose pulse to cells growing aerobically on a nonfermentable carbon source did not induce alcoholic fermentation, in spite of the remarkably high glucose uptake rates (Table 1).

![Diagram of metabolite flux distributions in Pichia anomala CBS 1984](image)

**Figure 1.** Metabolite flux distributions in *Pichia anomala* CBS 1984. The numbers represent the percentage of carbon passing through each reaction. The upper numbers represent flux distribution for an aerobic culture, whereas the lower numbers are those of an oxygen-limited culture. Reproduced from Fredlund et al. (2004), with permission.

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high activities of the fermentative enzymes pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH). Considering the minor formation of ethanol during aerobic conditions in spite of high enzyme activities, the yeast resembles Kluyveromyces lactis (Kiers et al., 1998), but differs from Pichia stipitis, which has very low ADH and PDC activities under aerobic conditions (Passoth et al., 1996). Under aerobic conditions, about 50% of the carbon that went through the PP pathway re-entered the glycolysis as fructose-6-phosphate or triose-3-phosphate (Fig. 1). This indicates that the PP pathway plays a more prominent role in Crabtree-negative yeasts as compared with Crabtree-positive yeasts and its activity has been shown to be correlated to the biomass yield (e.g. Bruinenberg et al., 1983; Blank et al., 2005). In P. anomala, the major part of the pyruvate entered the mitochondria and was metabolized via the citric-acid cycle, which operated as a cycle under these conditions (Fredlund et al., 2004a).

Oxygen limitation induced alcoholic fermentation and activated the enzymes of the fermentative pathway. The cells started to produce ethanol (6.2 mmol g⁻¹ h⁻¹) and glycerol (0.61 mmol g⁻¹ h⁻¹) and the specific glucose uptake rate increased more than twofold compared with the aerobic uptake rate. This demonstrated that P. anomala shows a clear Pasteur effect (Fredlund et al., 2004a). The metabolic flux distribution under oxygen limitation changed to a pattern similar to that of fermentative S. cerevisiae (Gombert et al., 2001; Fiaux et al., 2003). The production of reduced metabolites, mainly ethanol and glycerol, increased significantly. The PP pathway proportion decreased to 10% of the total glucose-6-phosphate breakdown and the citric-acid cycle operated as a two-branched pathway (Fredlund et al., 2004a).

The activities of ADH and PDC were remarkably high during aerobic growth on glucose. The ADH activity during growth on the nonfermentable carbon source succinate was substantially higher than in glucose-grown cells and decreased when glucose was added. There was also substantial PDC activity during growth on succinate, which was moderately up-regulated after glucose addition (Fredlund et al., 2004a). Transcriptional investigations (Fredlund, 2004) indicate that there are at least two ADHs involved in the ADH activities under the different culture conditions. One ADH appears to be responsible for ethanol consumption, aerobic activity during growth on glucose and ethanol formation during oxygen limitation. Another ADH seems to be responsible for the activity on succinate. The dual function of ethanol formation and consumption by one ADH has also been shown in the respiratory yeast P. stipitis (Cho & Jeffries, 1998; Passoth et al., 1998).

Pichia anomala contains a gene encoding an alternative cyanide-insensitive oxidase (Sakajo et al., 1991, 1999). Cyanide-insensitive respiration in P. anomala is induced by inhibitors of respiration and several other stresses (Veiga et al., 2003). The physiological function of this pathway is not clear. However, it has frequently been observed in Crabtree-negative yeasts and might be involved in the adjustment of energy available to the cell (Veiga et al., 2000, 2003).

Biotechnological importance

Pichia anomala in food and beverages

Due to the ability of Pichia anomala strains to survive and grow under high osmotic pressure and low pH (e.g. Kagiyama et al., 1988; Fredlund et al., 2002), it is a common spoilage organism in dairy and baking products, beer, high-salt environments and silage (Kagiyama et al., 1988; Kita-moto et al., 1999; Bonjean & Guillaume, 2003; Dufour et al., 2004a).
Pichia anomala has demonstrated antifungal activity towards a variety of fungi in different habitats (Passoth & Schnürer, 2003). Botrytis cinerea was inhibited on apple and grapevine, probably by secreted cell wall–lytic enzymes (Jijakli & Lepoivre, 1998; Masih et al., 2002; Santos et al., 2004). However, disrupting the PaEXG2 gene that encodes an extracellular exoglucanase did not impair the biocontrol activity of the manipulated P. anomala strain (Jijakli & Lepoivre, 1998). This suggests that the antifungal activity of P. anomala is due to more than one factor.

Pichia anomala has also been used to produce ribonucleotides and nucleosides that are applied as flavour enhancers and that are supposed to exhibit various therapeutic and immunostimulatory effects (Kim et al., 2002; Lee et al., 2004).

Antifungal activity of Pichia anomala

Pichia anomala has demonstrated antifungal activity towards a variety of fungi in different habitats (Passoth & Schnürer, 2003). Botrytis cinerea was inhibited on apple and grapevine, probably by secreted cell wall–lytic enzymes (Jijakli & Lepoivre, 1998; Masih et al., 2002; Santos et al., 2004). However, disrupting the PaEXG2 gene that encodes an extracellular exoglucanase did not impair the biocontrol activity of the manipulated P. anomala strain (Jijakli & Lepoivre, 1998). This suggests that the antifungal activity of P. anomala is due to more than one factor.

Pichia anomala has shown a strong biocontrol activity against a variety of moulds when grown on different cereal grains in both laboratory- and medium-scale silo experiments (Björnberg & Schnürer, 1993; Petersson & Schnürer, 1995, 1998; Petersson et al., 1998, 1999; Boysen et al., 2000; Druvefors et al., 2002; Druvefors & Schnürer, 2005). Druvefors & Schnürer (2005) found that P. anomala was unsurpassed among 60 different yeast species in the ability to inhibit growth of Penicillium roqueforti in a test tube version of airtight grain silos. Several yeasts grew to the same levels as P. anomala without having any mould inhibitory effect.

This argues against competition for space and nutrients as the main inhibitory mechanism of P. anomala. Moreover, addition of nutrients in a laboratory-scale biocontrol experiment did not impair the biocontrol activity of P. anomala. Addition of glucose even increased the antifungal effect of P. anomala [J121, without changing yeast biomass. Glucose did not inhibit the test fungus (Pen. roqueforti) when added to cultures without yeasts. This suggested that a product from glucose metabolism was responsible for mould inhibition (Druvefors et al., 2005). Further investigations showed that the decrease of fungal growth was accompanied by an increase of the ethyl acetate concentration, which was also shown to have antifungal activity (Fredlund et al., 2004c; Druvefors et al., 2005). When the haploid P. anomala strain CBS 1984 was cultivated at a water activity (aw) of 0.95, it produced less ethyl acetate than at aw 0.98 and had a diminished capacity to inhibit Pen. roqueforti (Fig. 3) (Fredlund et al., 2004c). Other mechanisms of fungal inhibition on grain may be due to the production of ethanol, oxygen consumption and CO2 production (Druvefors, 2004; Druvefors et al., 2002, 2005).

Pichia anomala killer toxins also have a potential against unwanted moulds and yeasts (e.g. Walker et al., 1995). The activity of P. anomala killer toxins against wine spoilage yeasts has recently been demonstrated (Comitini et al., 2004).

Clinical importance

Pichia anomala as an opportunist pathogen

As discussed before, Pichia anomala belongs to the microbial flora of a variety of foods and animal feed. However, it is not regarded as a health risk, as yeasts are not known to produce mycotoxins (Fleet, 1992). Pichia anomala is classified as a nonpathogenic, biosafety class-1 organism, without any restriction on handling and without any risk to healthy persons (DeHoog, 1996). However, more and more yeast species seem to be involved in clinical outbreaks in immunocompromised hosts (Hazen, 1995), including Saccharomyces cerevisiae (Murphy & Kavanagh, 1999) and P. anomala. In most cases, infections occurred after immuno-suppression, use of intravenous devices and previous treatment with antibacterial drugs (Haron et al., 1988). The growing occurrence of yeast infections might be due to the increasing number of immunocompromised patients rather than to higher virulence of the different yeasts (Hazen, 1995; Murphy & Kavanagh, 1999).

Potential use of Pichia anomala killer toxins for antimicrobial therapy

The therapeutic use of P. anomala killer toxins against yeast infections has been considered. As killer proteins themselves
are too big to be used in a therapeutic treatment, anti-idiotypic antibodies mimicking the action of killer toxins have been generated. Anti-idiotypic antibodies reflecting a *P. anomala* killer toxin have been demonstrated to exert a broad antimicrobial spectrum and were successfully used to treat model candidiasis in rat (Magliani *et al*., 1997, 2002).

**Perspectives**

*Pichia anomala* can inhibit harmful microorganisms in a variety of habitats. Thus, it has a technological potential as a biocontrol agent (Passoth & Schnürer, 2003). When growing on feed grain, it can add value to the feed apart from the antimould effect, as yeasts are regarded as sources of protein and vitamins (Litchfield, 1983). The yeast could also help to decrease the release of nutrients or greenhouse gases into the environment. Due to its ability to assimilate a broad range of nitrogen compounds, it can convert nitrate and ammonium compounds to proteins that can be used as a feedstock (Mo *et al*., 2004). Furthermore, it has a considerable phytase (myoinositol hexaphosphate phosphohydrolase, E.C. 3.1.3.8. and 3.1.3.26) activity. This enzyme is able to digest phytate, the major phosphate storage compound in plants, making it available to animal nutrition. Thus, *P. anomala* can improve the nutritional value of the feed and decrease the environmental burden by decreasing the phosphor content of the manure (Vohra & Satyanarayana, 2004).

Understanding the role of *P. anomala* in its different habitats requires a more extensive investigation of its physiology and genetics. The first attempts have been undertaken to reach a global understanding of metabolic regulation (Fredlund *et al*., 2004a). Further investigations of this respiratory yeast might lead to an extension of the use of yeasts as model organisms to understand the regulation of fundamental cellular processes.

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