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Genetics

A yeast model for the study of Batten disease

(*Saccharomyces cerevisiae*/*CLN3*/neuronal ceroid-lipofuscinoses)

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ABSTRACT **Although the *CLN3* gene for Batten disease, the most common inherited neurovisceral storage disease of childhood, was identified in 1995, the function of the corresponding protein still remains elusive. We previously cloned the *Saccharomyces cerevisiae* homologue to the human *CLN3* gene, designated *BTN1*, which is not essential and whose product is 39% identical and 59% similar to Cln3p. We report that *btn1*-Δ deletion yeast strains are more resistant to D-(−)-threo-2-amino-1-[*p*-nitrophenyl]-1,3-propanediol (denoted ANP), a phenotype that is complemented in yeast by the human *CLN3* gene. Furthermore, the severity of Batten disease in humans and the degree of ANP resistance in yeast are related when the equivalent amino acid replacements in Cln3p and Btn1p are compared. These results indicate that yeast can be used as a model for the study of Batten disease.**

The neuronal ceroid-lipofuscinoses (NCL) are the most common group of progressive neurodegenerative diseases in children, with an incidence as high as one in 12,500 live births, and with about 440,000 carriers in the USA (1, 2). The main features of this disease are failure of psychomotor development, impaired vision, seizures, familial occurrence, and premature death. NCL diseases traditionally have been divided into four subtypes based on the onset and clinical course of the disease, and are denoted by the following *CLN* genes responsible for the diseases: infantile, *CLN1* (Haltia-Santavuori disease); late infantile, *CLN2* (Jansky-Bielschowsky disease); juvenile, *CLN3* (Batten disease or Spielmeyer-Sjogren disease); and the rare adult, *CLN4* (Kufs disease) (2–8).

The NCL are characterized by the accumulation of autofluorescent hydrophobic material in the cytoplasm of neurons, and, to a lesser extent, many other cell types. More specifically, these deposits have been localized to lysosomes (9, 10). The ultrastructure of the electron-dense storage material varies, being granular in *CLN1* (9), curvilinear and fingerprint-like in different variant forms of *CLN2* (11), and heterogeneous in *CLN3* and *CLN4* (12). Furthermore, protein sequencing and immunological studies have revealed that subunit *c* of the mitochondrial ATP synthase is the major component of the storage material in *CLN2*, *CLN3*, and *CLN4*, but not in *CLN1* (13, 14). Accumulation of mitochondrial ATP synthase subunit *c* is not a result of increased expression of the P1 and P2 nuclear genes that encode the protein, nor does the stored protein have a different encoded sequence from that for normal individuals (15, 16). Furthermore, slower degradation of mitochondrial ATP synthase was found to occur in NCL fibroblasts as compared with normal cells. Although initially located in the mitochondria, mitochondrial ATP synthase subunit *c* accumulated in lysosomes of NCL cells, whereas the degradation of another inner mitochondrial membrane protein, cytochrome oxidase subunit IV, was unaffected, with no lysosomal accumulation (17, 18). However, a more recent study suggested that the accumulation of mitochondrial ATPase subunit *c* is a common unspecific phenomenon, which for unknown reasons is increased in NCL (19).

The *CLN3* gene, which is responsible for Batten disease, was deduced to encode a predicted protein of 438 amino acids (20). The majority of affected individuals carry a 1.02 kilobase deletion, which produced a frameshift mutation that leads to a predicted translation product of 181 amino acids, of which only the first 153 residues corresponded to the wild-type Cln3p (20). Although the *CLN3* gene has been clearly shown to be responsible for Batten disease, immunological studies of subcellular fractions led to the conclusion that Cln3p was localized to mitochondria (21), whereas confocal microscopic studies indicated that Cln3p was associated with lysosomes (22).

We previously identified the *Saccharomyces cerevisiae* homologue to the human *CLN3* gene, designated *BTN1*, which is 39% identical and 59% similar to Cln3p, and demonstrated that it is not essential and does not play a role in the degradation of mitochondrial ATPase subunit *c* (23). We report herein that an extensive search for a phenotype led to the finding that *btn1*-Δ deletion yeast strains were resistant to D-(−)-threo-2-amino-1-[*p*-nitrophenyl]-1,3-propanediol (denoted ANP) (Fig. 1), allowing yeast to serve as a model for Batten disease. This ANP resistance was complemented by the human *CLN3* gene. In addition, the degree of ANP resistance in yeast was related to the severity of Batten disease when the amino acid replacements of the Cln3p missense mutations were introduced in Btn1p.

MATERIALS AND METHODS

General Methods. General methods used in the construction of plasmids, restriction enzyme digests, separation of plasmid DNA and restriction fragments on agarose gels, ligation of DNA fragments, the isolation of plasmid DNA (24), and transformation of *Escherichia coli* (25) have been described. The cultivation, manipulation (26), and transformation (27) of yeast strains followed standard procedures.

Plasmids. The plasmids used in this study are listed in Table 1. Plasmid pAB1795 was constructed by ligating the *BTN1* containing *Eco*RI fragment of pAB1759 (23) with the *Eco*RI fragment of the 2-μ *TRP1* multicopy plasmid pAB1037. Similarly, a cDNA clone of human *CLN3* also was ligated to pAB1037, resulting in pAB1796. Point mutations L44P, L112P, E243K, V289C, R293C and R293H in the *BTN1* gene were generated, as described (28), in a single-copy plasmid pAB1793 (*CEN6 URA3 BTN1*), resulting in, respectively, plasmids pAB2040–pAB2045.

Yeast Strains. The isogenic *btn1*-Δ yeast strains B-10195 (*MAT***a** *btn1*-Δ::*HIS3 CYC1*$^+$ *cyc7*-Δ::*CYH2 leu2,3-112 ura3–52 his3*-Δ*1 trp1–289*) (denoted *btn1*-Δ *CYC1*$^+$) and B-10196

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Abbreviations: ANP, D-(−)-threo-2-amino-1-[*p*-nitrophenyl]-1,3-propanediol; NCL, neuronal ceroid-lipofuscinoses; YPD, yeast glucose medium; YPG, yeast glycerol medium.

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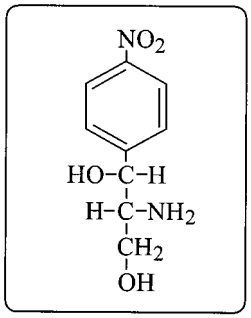

FIG. 1. Structure of ANP.

(*MAT***a** *btn1*-Δ::*HIS3 cyc1*-Δ::*lacZ cyc7*-Δ::*CYH2 leu2,3–112 ura3–52 his3*-Δ*1 trp1–289*) (denoted *btn1*-Δ *cyc1*-Δ) were derived from B-7553 (*MAT***a** *BTN1*$^+$ *CYC1*$^+$ *cyc7*-Δ::*CYH2 leu2,3–112 ura3–52 his3*-Δ*1 trp1–289*) (denoted *BTN1*$^+$ *CYC1*$^+$) and B-6748 (*MAT***a** *BTN1*$^+$ *cyc1*-Δ::*lacZ cyc7*-Δ::*CYH2 leu2,3–112 ura3–52 his3*-Δ*1 trp1–289*) (denoted *BTN1*$^+$ *cyc1*-Δ), respectively, by gene disruption (29). The *cyc1*-Δ *cyc7*-Δ strains are defective in respiration because of the absence of the isocytochromes *c* encoded by *CYC1* (30) and *CYC7* (31). Similarly, B-10198 (*MAT***a** *btn1*-Δ *cyc1*-Δ *cyc7–67 GAL2 trp1–289 his3*-Δ*1 ura3–52*) was derived from B-10197 (*MAT***a** *cyc1*-Δ *cyc7–67 GAL2 trp1–289 his3*-Δ1 *ura3–52*) by gene disruption. B-10199–B-10201 were derived from B-10198 by introducing the plasmids, respectively, pAB1037, pAB1795, and pAB1796 (Table 1). The strains B-11328–B-11333, containing the alterations listed below and in Table 1, were derived from B-10196 (*MAT***a** *btn1*-Δ *cyc1*-Δ *cyc7–67 trp1–289 his3*-Δ*1 ura3–52*) by introduction of the plasmids pAB2040–pAB2045.

Growth Media. Yeast media YPD (glucose medium) and YPG (glycerol medium) were prepared as described (26), with ANP added at the indicated concentrations after autoclaving. Growth in liquid media was measured by using a Klett Summerson Photoelectric Colorimeter.

RESULTS AND DISCUSSION

***Btn1*-Δ Strains Are Resistant to ANP.** To uncover a phenotype and to provide insight of the function of Btn1p, the following isogenic yeast strains were constructed: B-7553 (*BTN1*$^+$ *CYC1*$^+$); B-10195 (*btn1*-Δ *CYC1*$^+$); B-6748 (*BTN1*$^+$ *cyc1*-Δ); and B-10196 (*btn1*-Δ *cyc1*-Δ).

In addition, all of these strains lack iso-2-cytochrome *c* because of the *cyc7*-Δ deletion, and the *cyc1*-Δ *cyc7*-Δ strains are defective in respiration because of the absence of both isocytochromes *c*. These *BTN1*$^+$ and *btn1*-Δ sets of isogenic yeast strains were tested with more than 100 different types of media and growth conditions. No differences were observed with many conditions, including those that are associated with mitochondrial or vacuolar defects, some of which have been described recently by Hampsey (32). Many compounds were added to media at different concentrations, such that sensitivity or resistance could be revealed. Potential differences in growth for *BTN1*$^+$ and *btn1*-Δ strains for each compound were monitored on a fermentable carbon source, glucose and nonfermentable carbon source, glycerol. The rationale for this was that if accumulation of mitochondrial ATPase subunit *c* in the lysosome was caused by defective mitochondria, then a phenotype might be more obvious while the yeast cells are growing by respiration, rather than fermentation. In addition the *cyc1*-Δ *cyc7*-Δ strains may uncover a phenotype, because of their defect in respiration. One of these compounds, ANP (Fig. 1), initially was uncovered because it is a breakdown product of chloramphenicol and previously was shown to preferentially inhibit the growth of *anp1*-Δ strains lacking the *ANP1* gene product (33). *Btn1*-Δ strains were found to be preferentially resistant to elevated concentrations of ANP in growth media. The ANP resistance of *CYC1*$^+$ *btn1*-Δ strains was more clearly revealed when tested on media containing a nonfermentable carbon source, such as glycerol (YPG), rather than on the fermentable carbon source, glucose (YPD). Also, ANP resistance of *btn1*-Δ was even more clearly revealed with *cyc1*-Δ strains when tested both on solid (Fig. 2) and liquid (Fig. 3*A*) glucose medium (YPD). The dependence of this ANP resistance on the absence of Btn1p was confirmed by reintroducing *BTN1* on a plasmid (Table 1). In fact, the ANP resistance of *btn1*-Δ strains could be complemented by a multicopy P_{GAL1}-*BTN1* plasmid on glucose medium, without galactose induction, indicating that the basal uninduced level was sufficient.

Functional Equivalence of Btn1p and Cln3p. Most importantly, by introducing a plasmid containing the human *CLN3*

Table 1. Plasmids used in this study

Plasmid number	Genotype	Amino acid replacement in Btn1p
pAA1037	2 μ *TRP1*	—
pAB1795	2 μ *TRP1* P_{GAL1}-*BTN1*	—
pAB1796	2 μ *TRP1* P_{GAL1}-*CLN3*	—
pAA625	*CEN6 URA3*	—
pAB1793	*CEN6 URA3 BTN1*	—
pAB2040	*CEN6 URA3 btn1-1*	L44P
pAB2041	*CEN6 URA3 btn1-2*	L112P
pAB2042	*CEN6 URA3 btn1-3*	E243F
pAB2043	*CEN6 URA3 btn1-4*	V289F
pAB2044	*CEN6 URA3 btn1-5*	R293C
pAB2045	*CEN6 URA3 btn1-6*	R293H

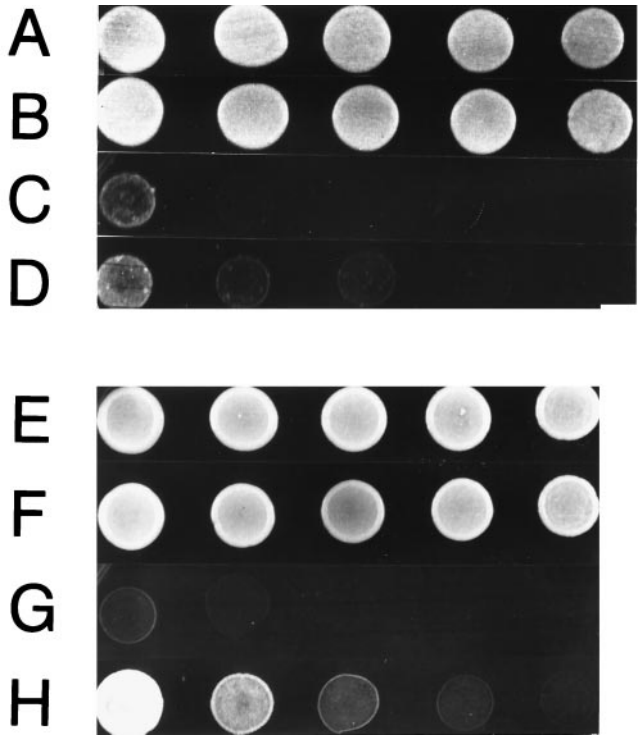

FIG. 2. Growth at 30°C of *BTN1*$^+$ and *btn1*-Δ strains on YPG containing 1.33 mg/ml of ANP, and YPD containing 2 mg/ml of ANP. (*A*) B-7553 (*BTN1*$^+$ *CYC1*$^+$), YPG, 4 days. (*B*) B-10195 (*btn1*-Δ *CYC1*$^+$), YPG, 4 days. (*C*) B-7553 (*BTN1*$^+$ *CYC1*$^+$), YPG + ANP, 7 days. (*D*) B-10195 (*btn1*-Δ *CYC1*$^+$), YPG + ANP, 7 days. (*E*) B-6748 (*BTN1*$^+$ *cyc1*-Δ), YPD, 4 days. (*F*) B-10196 (*btn1*-Δ *cyc1*-Δ), YPD, 4 days. (*G*) B-6748 (*BTN1*$^+$ *cyc1*-Δ), YPD + ANP, 7 days. (*H*) B-10196 (*btn1*-Δ *cyc1*-Δ), YPD + ANP, 7 days. Serial dilutions (1/10) of the strains were spotted on the various media.

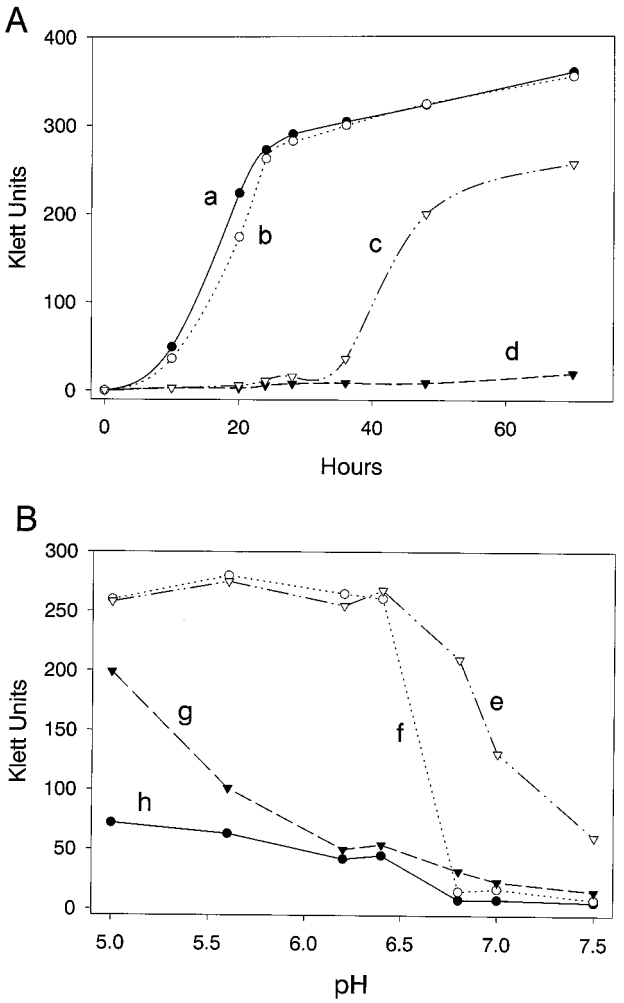

FIG. 3. (*A*) Growth curves of *BTN1*$^+$ and *btn1*-Δ strains in YPD and YPD containing 0.66 mg/ml of ANP. (*a*) B-6748 (*BTN1*$^+$ *cyc1*-Δ), YPD. (*b*) B-10196 (*btn1*-Δ *cyc1*-Δ) in YPD. (*c*) B-10196 (*btn1*-Δ *cyc1*-Δ) in YPD + ANP. (*d*) B-6748 (*BTN1*$^+$ *cyc1*-Δ) in YPD + ANP. (*B*) Growth of *BTN1*$^+$ and *btn1*-Δ strains in YPD containing 0.66 mg/ml of ANP and at various initial pH values. (*e*) B-10196 (*btn1*-Δ *cyc1*-Δ), 48 hr. (*f*) B-6748 (*BTN1*$^+$ *cyc1*-Δ), 48 hr. (*g*) B-10196 (*btn1*-Δ *cyc1*-Δ), 24 hr. (*h*) B-6748 (*BTN1*$^+$ *cyc1*-Δ), 24 hr.

cDNA, we demonstrated that the human Cln3p also re-establishes the yeasts' sensitivity to ANP, indicating that this human protein functions in yeast (Table 2). Complementation was observed on glucose medium, identical to the results observed with the P_{GAL1}-*BTN1* plasmid. Similar results were also observed for a *btn1*-Δ *CYC1*$^+$ yeast strain grown on glycerol medium (YPG) (data not presented). This functional equivalence allows us not only to investigate Btn1p, but also Cln3p in *S. cerevisiae*.

Of 139 individuals with Batten disease, 74% were found to be homozygous for a 1.02-kilobase deletion, which results in a truncated protein that contains only the first 153 amino acids of the normal Cln3p (20, 34). Six missense mutations, which occur heterozygous with the 1.02-kilobase deletion, also have been characterized. Some of these individuals have less severe forms of Batten disease (34). The residues that were altered in these missense mutant forms are conserved between Btn1p and Cln3p.

To compare the degree of ANP resistance in yeast to the severity of Batten disease in humans, the amino acid replacements of the Cln3p missense mutations were introduced in Btn1p. The results, summarized in Table 3, demonstrated a correlation of the severity of Batten disease and the ANP resistance with the corresponding amino acid replacements. The *btn1*-Δ mutation can be considered to be equivalent to the common, most severe form of Batten disease caused by the 1.02-kilobase deletion. The L170P and E295K Cln3p replacements are described as causing nonclassical forms of Batten disease, being symptomatically dominated by only visual failure, whereas the corresponding Btn1p L112P and E243K replacements did not cause resistance to ANP. On the other hand, the Cln3p replacements V330F, R334C, and R334H, cause a severe classical form of Batten disease, and the corresponding Btn1p replacements produced partial resistance to ANP. Although we observe a general correlation of ANP resistance to the severity of Batten disease associated to these point mutations, any future conclusions must take into consideration the variability that different individuals show with regard the onset of Batten disease. Such variation may be a result of genetic or environmental factors (34). A particular mutation cannot be used to predict the disease phenotype.

ANP Resistance Is pH Dependent. The growth of the *BTN1*$^+$ *cyc1*-Δ and *btn1*-Δ *cyc1*-Δ strains in YPD and YPD + ANP media is shown in Fig. 3*A*. Further studies prompted us to alter the initial pH of the YPD + ANP media (Fig. 3*B*). At the initial pH of unadjusted YPD and YPG, approximately pH 6.7, the *BTN1*$^+$ *cyc1*-Δ and *btn1*-Δ *cyc1*-Δ strains are readily distinguished. After slow growth within the first 24 hr for both *BTN1*$^+$ and *btn1*-Δ strains, the *btn1*-Δ strain grew 10-fold more after 48 hr. By adjusting the initial pH to below and above 6.5, ANP is, respectively, less and more toxic, leading to diminished differential responses. The growth in media containing ANP is complicated by the changes of pH during cultivation. Furthermore, ANP resistance of *btn1*-Δ strains is not revealed in

Table 2. Complementation of ANP resistance in *btn1*-Δ strains by plasmids containing the yeast *BTN1* and human *CLN3* genes

	(*BTN1*$^+$ *cyc1*-Δ)	(*btn1*-Δ *cyc1*-Δ)			
	B-10197	B-10198	B-10200	B-10201	B-10199
ANP, mg/ml	— (No plasmid)	— (No plasmid)	p[*BTN1*] (pAB1795)	p[*CLN3*] (pAB1796)	No insert (pAB1037)
0	5	5	5	5	5
1.00	2	5	3	3	5
1.33	1	5	2	2	5
1.66	1	5	1	1	4
2.00	0	5	1	1	4

All yeast strains, B-10197–B-10201, are *GAL2*, and the *BTN1* and *CLN3* genes in the p[*BTN1*] and p[*CLN3*] plasmids are under the control of the *GAL1* promoter. The strains were grown for 2 days on synthetic galacose medium lacking tryptophan, and subsequently tested on YPD containing various concentrations of ANP. Relative growth is indicated by 0 (no growth) to 5 (normal growth). The expression of the human gene in yeast is demonstrated, for example, by diminished growth of B-10201 on medium containing 1.66 mg/ml of ANP.

Table 3. The relationship between the severity of Batten disease (34) and ANP resistance in yeast with corresponding alterations

Cln3p (human)		Btn1p (yeast)	
Alteration	Batten disease severity	Alteration	ANP resistance
Deletion	+++	Deletion	+++
L101P	++	L44P	++
L170P	+	L112P	0
E295K	+	E243K	0
V330F	+++	V289F	+
R334C	+++	R293C	++
R334H	+++	H293H	++
None	0	None	0

B-10196 (*btn1*-Δ *cyc1*-Δ) was transformed with single copy plasmids, and the growth of the resulting strains was determined on YPD containing 2 mg/ml of ANP.

buffered media unless the initial pH is low enough to support growth of both *BTN1*$^+$ and *btn1*-Δ strains.

Although the mechanisms for the differential response of *BTN1*$^+$ and *btn1*-Δ strains to ANP, and the greater distinction with respiratory deficient strains are unknown, the phenotype allows us to investigate gene products interacting with Btn1p by using the many genetic approaches conveniently carried out with yeast (35). The lack of Anp1p in *anp1*-Δ strains causes yeast to be abnormally sensitive to ANP (33). Further characterization has shown that *anp1* mutants retain Mnt1p and the protease DPAP-A in the medial and late golgi, respectively (36, 37), and that Anp1p forms part of a complex located in the *cis* golgi possessing α-1,6-mannosyltransferase activity (38). Currently, there is no evidence that the sensitivity and resistance of *anp1*-Δ and *btn1*-Δ strains, respectively, are related. On the other hand, it is tempting to speculate that the influence of pH on ANP resistance is related to the report that Cln3p is localized to the lysosome (22) (which is denoted as a vacuole in yeast), a compartment associated with maintenance of pH (39).

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