

Comparison of the effects of *bax*-expression in yeast under fermentative and respiratory conditions: investigation of the role of adenine nucleotides carrier and cytochrome *c*

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Abstract A new system for *bax*-expression in yeast has been devised to investigate *bax*'s effect under fermentative and respiro-fermentative conditions. This has allowed us to show unambiguously that the ability of *bax* to kill yeast is higher under respiratory conditions than under purely fermentative conditions. The extent of killing under respiro-fermentative conditions (non-repressive sugars) is intermediate. It has been proposed that the two proteins adenine nucleotides carrier (ANC) and cytochrome *c* play a crucial role in *bax*-induced cell death. We have investigated the effects of deletion of the genes encoding the two proteins on the toxicity induced by *bax*, using this new system. The absence of ANC did not modify *bax*-induced lethality in any way. Moreover, the absence of cytochrome *c* also did not prevent *bax*-induced death. Only the kinetics of lethality were altered. All these effects are prevented by co-expression of *bcl-x_L*.

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Key words: *bax*-Expression; Adenine nucleotides carrier; Cytochrome *c*

1. Introduction

Expression of proteins of the *bcl-2* family, namely the proapoptotic protein *bax*, in the budding yeast *Saccharomyces cerevisiae* has been used by several groups to study the molecular mechanisms of action of these proteins [1–5]. Since yeast does not support any spontaneous apoptotic form of death, it represents a neutral background that could permit observations of molecular events that lead to *bax*-induced cellular death, independent of the complex regulation network present in mammalian cells. Yeast has been used to study the protective effect of anti-apoptotic proteins *bcl-2/bcl-x_L* on *bax*-mediated toxicity [2–4], the death phenomena caused by superoxide-dismutase inactivation independent on *bax* and the loss of viability in the stationary phase [6]. More recently, attempts to study the molecular effects of caspases in yeast have also been published [7,8].

The fact that *bax*-expression leads to death of yeast cells is now well-documented. One interesting aspect of *bax*-expression in yeast is that, as in mammalian cells that undergo apoptosis [9,10], several proteins are relocated from the mito-

chondrial intermembrane space to the cytosol. One of these proteins is cytochrome *c* [4], a soluble component of the mitochondrial electron transport chain.

Both the mechanism and the function of this relocation remain unclear. It has been demonstrated that exogenous cytochrome *c* was able to switch-on activation of caspases both in vivo [11,12] and in vitro [13]. However, several authors have reported observations of apoptotic cell death where cytochrome *c* was not relocated [14]. This may support the idea that, depending on the cell type and/or apoptotic stimulus, cytochrome *c* relocation may or may not be involved in cell death. In addition, it has to be noted that the universality of the model involving opening of the permeability transition pore (PTP) in cytochrome *c* relocation has been doubted by a number of observations: (a) delay between cytochrome *c* release and PTP-opening in apoptotic cells [15,16], (b) in vitro experiments showing possibilities of cytochrome *c* release without PTP-opening [17,18], (c) PTP-opening without cytochrome *c* release [19] and (d) total absence of any permeability transition following *bax*-expression in yeast [20]. Moreover, recent experiments have shown that in vitro activation of caspases following PTP-opening by atractyloside treatment of the mitochondria fraction could be explained by release of a peroxysomal protease, cathepsin B [21], which is inhibited by specific caspase inhibitors [22]. It appears to be more and more obvious that, depending on the apoptotic model, mitochondria may or may not be involved in the early steps of apoptosis. *Bax*-expressing yeasts could therefore be compared to several mammalian models where permeability transition appears to be a consequence, not a cause, of cytochrome *c* release and caspase activation, an idea supported by the fact that activated caspases are able to open PTP and can thus amplify the initial apoptotic signal [23].

Yeast is able to grow both under respiratory and fermentative conditions, making it a unique tool to assay the functional involvement of mitochondria in *bax*-effects. It has been shown that ρ_0 yeast cells are less sensitive to *bax*-induced death [3]. Unfortunately, the *GAL10*-driven expression system, which has been used by a number of groups, including ours, is somewhat difficult to manipulate under fermentative conditions since the *GAL10* promoter is strongly repressed by glucose. In addition, a number of respiratory-deficient yeast mutants grow badly with galactose as a carbon source, making 'glucose to galactose' shift experiments hardly interpretable. Thus, a number of experiments that emphasize the involvement of mitochondrial protein in *bax*-effects merely on the basis of resistance to *bax*-expression, under fermentative

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Abbreviations: ANC, adenine nucleotides carrier; PTP, permeability transition pore; VDAC, voltage-dependent anion channel

conditions, must be carefully evaluated to ensure that no side-effects explain the results. In this context, elucidation of the role of the F_0F_1 -ATPase subunits [24] and the adenine nucleotides carrier (ANC) [25] seems to be particularly important.

In this paper, we developed a new system for expressing *bax* and *bcl-x_L* in yeast, based on the bacterial tetracyclin-repressed promoter (tetracyclin-off system [26]). This system is insensitive to the nature of the carbon source present in culture media, making it a powerful tool to assay the dependence of *bax*-effects on different carbon sources (respiratory or fermentative) and also to test *bax*-sensitivity of specific respiratory-deficient mutants. For example, a direct involvement of PTP in *bax*-effects can be tested by expressing *bax* in yeast strains inactivated for putative PTP components. We have previously shown that the outer membrane voltage-dependent anion channel (VDAC) was not involved in *bax*-mediated effects, including relocation of cytochrome *c* [20]. Here, we have tested the involvement of the other putative major component of PTP, the inner-membrane ANC.

Finally, we were able to assay the effect of *bax*-expression and *bax/bcl-x_L* co-expression on the growth and death of a cytochrome *c*-less yeast strain to test the actual involvement of cytochrome *c* in *bax*-induced yeast cell death.

2. Materials and methods

2.1. Expression plasmids

The human *bax* gene, chemically synthesized with yeast codon bias, encodes the α -isoform of the protein [3] and was placed under the control of a tetracyclin-regulatable promoter (Enrique Herrero, Lleida University, Lleida, Spain) as follows: the *bax* gene, fused to a fragment containing the *SUC2* transcription terminator with a 5'-DNA sequence encoding the *c-myc* epitope EQKLOSEEDLNG, was isolated from a pSK+ plasmid as a *Bgl*II-*Pvu*II fragment and was then subcloned in centromeric plasmids carrying the *tetR* repressor and the *tetO* operator sequences [26]. Insertion was done between the *Hpa*I and *Bam*HI sites of pCM189 (carrying *URA3* as yeast selection marker) or between the *Stu*I and *Bam*HI sites of pCM184 (carrying *TRP1* as yeast selection marker).

The human *bcl-x_L* gene was subcloned under the control of a tetracyclin-regulatable promoter in two steps: the *bcl-x_L*-expression cassette, under the control of the *GAL10* promoter, was isolated as a *Sac*I-*Sal*I fragment from pDP83A described elsewhere [3] and inserted between the *Sac*I and *Sal*I sites of a pFL39 vector. The *Bcl-x_L* gene was then isolated from this construct by a *Mse*I digestion and inserted at the *Hpa*I site of pCM184 [26]. The orientation of the construction was checked by a *Hind*III digestion.

2.2. Yeast strains

Strains used are summarized in Table 1. The wild-type W303-1A strain transformed with pCM189-*bax* is referred to as WtB1, W303-1A strain transformed with pCM184-*bcl-x_L* is referred to as WtB3, W303-1A strain transformed with both pCM189-*bax* and pCM184-*bcl-x_L* is referred to as WtB4. Cytochrome *c*-less strain B-06748 (David Pearce, Rochester University, Rochester, USA) was transformed with pCM189-*bax* (CtB1 strain) or co-transformed with pCM189-*bax* and pCM184-*bcl-x_L* (CtB2 strain). ANC-less JL1-3-1A strain (Jordan Kolarov, Comenius University, Bratislava, Slovakia) was transformed with pCM184-*bax* and is referred to as AtB1. All transformations were controlled by PCR on yeast colonies.

2.3. Growth conditions and colony forming efficiency

Yeast cells were grown on 0.175% yeast nitrogen base, 0.5% ammonium sulfate, 0.1% KH_2PO_4 , 0.2% drop mix (a mixture containing 3 g of each auxotrophic marker except adenine, histidine, lysine, leucine, uracil and tryptophan) with addition of the supplements required for growth of the specific strain (SC-medium). The carbon source was either 2% glucose (fermentative growth, SC-glucose) or 2% D,L-lactate (respiratory growth, SC-lactate). In several experiments, other carbon sources such as 2% glycerol, 1% sucrose, 1% raffinose, 2% mannose or

2% galactose were used (see Section 3). In the presence of 1 $\mu\text{g}/\text{ml}$ doxycyclin, the tetracyclin-regulatable promoter was repressed. Expression of genes driven by such a promoter was induced upon antibiotic removal [26]. For survival tests, cells were grown in the same media as above and then spread onto non-selective YPD medium in the presence of doxycyclin (1% yeast extract, 1% bacto-peptone, 2% glucose, 10 $\mu\text{g}/\text{ml}$ doxycyclin).

2.4. Protein analysis

Cells were harvested after different induction times (see Section 3) and spheroplasts and mitochondria were prepared as described previously [27,28]. The cytochrome *c* content was measured by spectrophotometry on dithionite-reduced versus H_2O_2 -oxidized samples in an Aminco DW2000 double-beam spectrophotometer. The protein concentration was measured with a biuret method.

Bax-expression was analyzed after separating proteins by SDS-PAGE and blotting onto PVDF membranes (Problott, Applied Biosystems). Western analyses were done with a primary anti-*c-myc* antibody (Calbiochem) and a secondary anti-mouse-HRP antibody (Immunopure, Pierce) revealed by enhanced chemio-luminescence (ECL) (Amersham).

2.5. Electron microscopy

Spheroplasts were fixed with 2.5% glutaraldehyde in a 0.1 M sodium phosphate buffer (pH 7.2) for 4 h at 4°C, post-fixed with 1% osmic acid in the same buffer and stained with 2% uranyl acetate.

3. Results and discussion

3.1. Expression of *bax* and *bcl-x_L* under the control of a 'tetracyclin-off' system

Bax and *bcl-x_L* were placed under the control of a 'tetracyclin-off' expression system (see Section 2). With this system, we were able to induce *bax* and *bcl-x_L*-expression under fermentative conditions as well as under respiratory conditions (Fig. 1, top).

Cells were first grown in liquid medium in the presence of doxycyclin (expression-repressed) and then spread on glucose or glycerol-supplemented solid medium in the presence (expression-repressed) or in the absence (expression-induced) of doxycyclin. No cell was able to grow both on glycerol and glucose medium when *bax* was expressed (data not shown). The same experiment was done with cells carrying two plas-

Table 1

Yeast strains	Genotypes
W303-1B	MAT a, ade2, his3, leu2, trp1, ura3
WtB1 ^a	MAT a, ade2, his3, leu2, trp1, ura3, pCM189/tetp- <i>bax</i> (URA3)
WtB3 ^a	MAT a, ade2, his3, leu2, trp1, ura3, pCM184/tetp- <i>bcl-x_L</i> (TRP1)
WtB4 ^a	MAT a, ade2, his3, leu2, trp1, ura3, pCM189/tetp- <i>bax</i> (URA3), pCM184/tetp- <i>bcl-x_L</i> (TRP1)
B-06748 ^b	MAT a, his3, leu2, trp1, ura3, cyh2, cyc1::lacZ, cyc7::CYH2
CtB1 ^a	MAT a, his3, leu2, trp1, ura3, cyh2, cyc1::lacZ, cyc7::CYH2, pCM189/tetp- <i>bax</i> (URA3)
CtB2 ^a	MAT a, his3, leu2, trp1, ura3, cyh2, cyc1::lacZ, cyc7::CYH2, pCM189/tetp- <i>bax</i> (URA3), pCM184/tetp- <i>bcl-x_L</i> (TRP1)
JL1-3-1A ^c	MAT a, ade2, trp1, leu2, his3, ura3, anc1::LEU2, anc2::HIS3, anc3::URA3
AtB1 ^a	MAT a, ade2, trp1, leu2, his3, ura3, anc1::LEU2, anc2::HIS3, anc3::URA3, pCM184/tetp- <i>bax</i> (TRP1)
HT444/ <i>bax</i> ^d	MAT a, his4, trp1, lys2, leu2, bax-LEU2

^aThis study.

^bA gift from David Pearce (Rochester).

^cA gift from Jordan Kolarov (Bratislava).

^d[3].

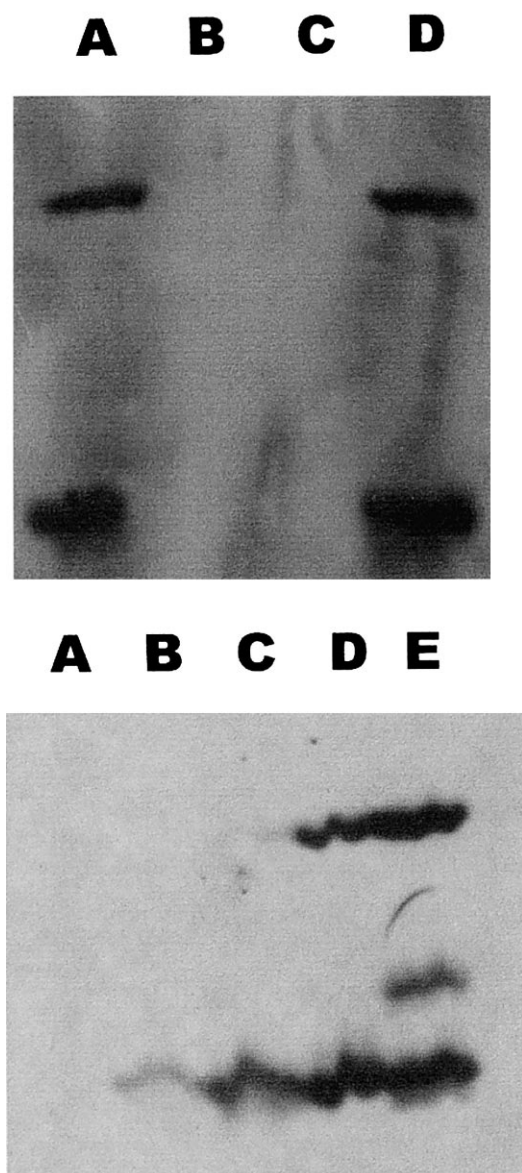


Fig. 1. *Bax*-expression. WtB1 cells were grown aerobically under conditions of *bax*-expression or absence of expression (see below). Spheroplasts were prepared, solubilized, 0.25 mg proteins were separated by SDS-PAGE, transferred onto PVDF membranes and revealed with a primary anti-*c-myc* antibody, a secondary anti-mouse antibody coupled to HRP and revealed by ECL. The upper band corresponds to native *bax* at about 21 kDa, the lower band corresponds to 17 kDa [29]. The intermediate band of 18.5 kDa observed in (bottom, lane E) may be observed only at very high levels of expression. Top: comparison of tetracyclin promoter-driven expression in lactate and glucose. Lane A: glucose in the absence of doxycyclin, lane B: glucose in the presence of doxycyclin, lane C: lactate in the presence of doxycyclin, lane D: lactate in the absence of doxycyclin. Bottom: comparison of tetracyclin promoter and *GAL10* promoter-driven *bax*-expression with lactate as a substrate. Lane A: WtB1 in the presence of doxycyclin, lane B: WtB1 after 4 h in the absence of doxycyclin, lane C: WtB1 after 12 h in the absence of doxycyclin, lane D: HT444/*bax* in complete medium after 3 h in the presence of galactose, lane E: HT444/*bax* SC-medium after 3 h in the presence of galactose.

mids, one encoding for *bax*, the other for *bcl-x_L*, both under the control of the tetracyclin-off promoter. The proportion of cells growing on plates upon induction was close to 100% of the cells growing on plates under repressive conditions, show-

ing that *bcl-x_L* can almost totally rescue *bax*-mediated effects in yeast (data not shown, see also below).

In order to allow for further comparisons of the results obtained with the *tetO*-driven expression system with those obtained previously with the *GAL10*-driven expression system, we compared the level of expression of *bax* under the control of both promoters. Fig. 1 (bottom) is a Western blot analysis of spheroplast proteins expressing *bax* under the control of the two promoters. As reported previously [29], *bax* expressed under the control of the *GAL10* promoter appears in two forms: the expected 21 kDa native protein and a shorter form of about 17 kDa generated from a N-terminal cleavage of the protein. Western blot analysis of spheroplasts expressing *bax* under the control of the *tetO* promoter allowed two conclusions: (i) the total amount of *bax* (short form+native form) is markedly lower than with the *GAL10* promoter-driven system and (ii) the relative amount of the short form is much higher than in the *GAL10* promoter-driven system. This result indicated that *bax*-induced growth arrest was not affected by the N-terminal cleavage of the protein nor by a lower amount produced in the tetracyclin-off expression system.

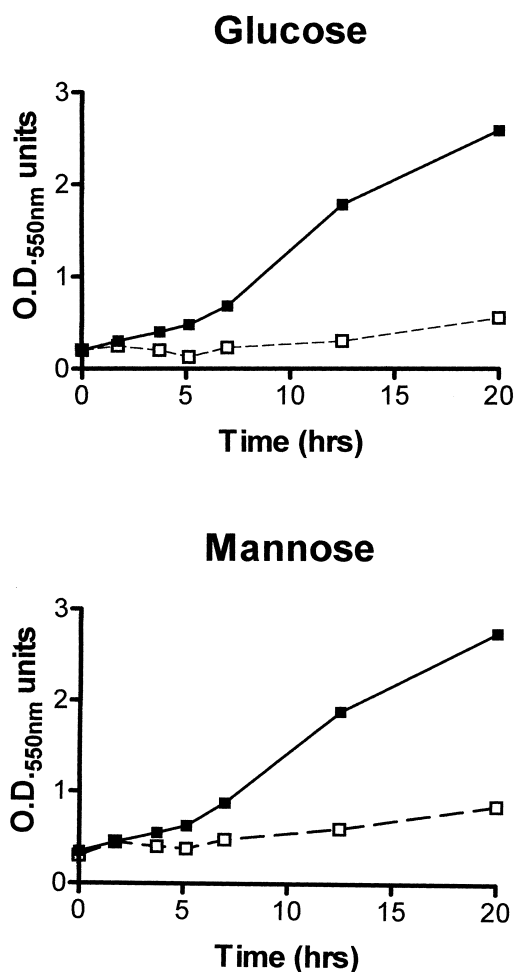


Fig. 2. *Bax*-induced growth arrest. WtB1 cells were grown aerobically in SC-medium supplemented with the indicated sugar, in the presence of doxycyclin until the early exponential growth phase ($OD_{550\text{ nm}} = 1$), then harvested, washed twice and resuspended at 0.1 $OD_{550\text{ nm}}$ U in the same medium in the absence (open squares) or in the presence (full squares) of doxycyclin.

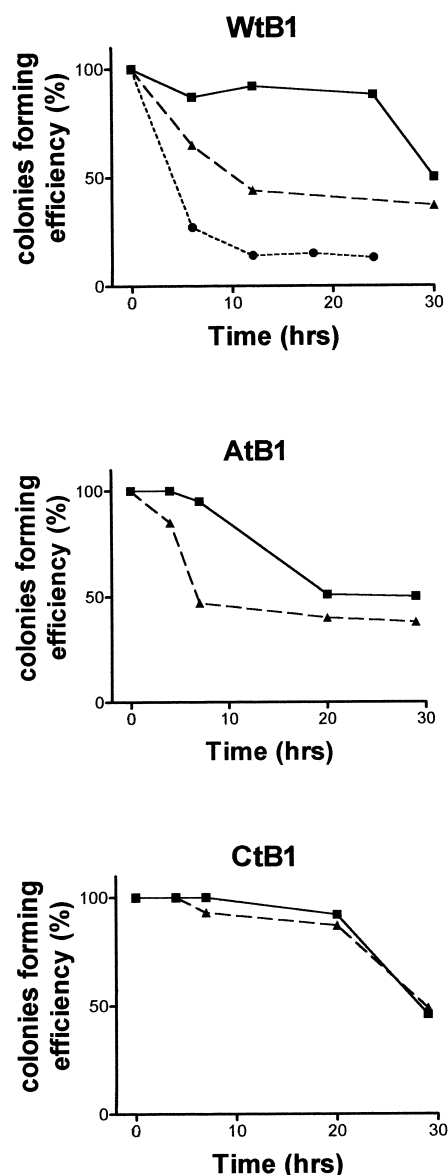


Fig. 3. *Bax*-induced lethality. Cultures were made in the presence of glucose (squares), mannose (triangles) or lactate (circles) in the presence of doxycyclin and then transferred in the same medium in the absence or in the presence of doxycyclin. At different times, an aliquot of the cultures was taken out and spreaded at 200 cells/plate on a complete glucose solid medium in the presence of doxycyclin. The Y-axis is the % of colonies appearing on plates done from the (–doxycyclin) culture over the number of colonies appearing on plates done from the (+doxycyclin) culture. Top: WtB1 strain, middle: AtB1 strain, bottom: CtB1 strain.

3.2. Effect of the carbon source on *bax*-induced growth arrest and mortality

From experiments reported previously, *bax* was as efficient in inducing growth arrest upon glycerol as upon glucose media. But these experiments in solid media do not allow us to draw any conclusion about the kinetics of *bax*-induced growth arrest and mortality.

Therefore, cells were grown in liquid medium in the presence of doxycyclin (no expression) and then inoculated to a liquid medium without doxycyclin (*bax*-expression-induced). At different time-points, aliquots were taken to measure the

colony forming efficiency (on non-selective doxycyclin-containing plates). This experiment was done in medium supplemented with lactate or glucose or sugars which do not induce a strong catabolic repression of respiratory enzymes (namely galactose, raffinose, mannose and sucrose).

Fig. 2 depicts the optical density (OD) of cultures after a shift to medium without doxycyclin with glucose (top) or mannose (bottom) as substrates. It can be seen that the growth rate was strongly reduced (although not fully abolished) following the shift. Identical results were obtained with other carbon sources (lactate, raffinose, sucrose and galactose) (data not shown). This demonstrates that the type of metabolism (respiratory or fermentative, repressive or not) did not influence *bax*-induced growth arrest.

Fig. 3 (top) reports the colony forming efficiency of the *bax*-expressing wild-type strain WtB1, after a shift to medium without doxycyclin. A clear difference was evidenced between lactate and glucose-containing medium. Cell death occurred rapidly in lactate medium since only 10–15% of the cells were able to form colonies after a 12 h induction. In contrast, almost all cells were viable after a 20 h induction in glucose medium and about 50% of the cells was still able to form colonies after a 30 h induction. A 48 h induction was required to reach a viability level as low as in lactate medium (not shown). Importantly, with sugars which do not induce catabolic repression of respiratory enzymes, such as mannose, cells were significantly less resistant to *bax*-effects than glucose-grown cells, but remained more resistant than lactate-grown cells.

From this set of data, it can be concluded that the type of metabolism did not affect *bax*-induced growth arrest. This occurred rapidly both in fermentative medium and in respiratory medium. On the contrary, the type of metabolism strongly affected *bax*-induced mortality, which ‘follows’ growth arrest under respiratory conditions, but is markedly delayed under purely fermentative conditions (glucose). Under respiro-fermentative conditions (other sugars), the situation is intermediate, suggesting that one or more protein(s) repressed by glucose are involved in the kinetics of the *bax*-induced killing effect.

3.3. *Bax*-induced growth arrest and lethality in an *ANC*-less strain

A major point of discussion about *bax*-induced cell death in yeast and, more generally, about the involvement of mitochondria in apoptosis is the exact role of the so-called PTP. On the basis of in vitro experiments, Kroemer and colleagues, together with other groups, propose that PTP-opening is an early and crucial step that directly induces cytochrome *c* release (and other apoptogenic factors such as apoptosis inducing factor), allowing for further activation of apoptotic pathways, namely the caspase cascades [30]. However, other groups working on in vivo systems did not observe any obvious time correlation between PTP-opening (measured by mitochondrial $\Delta\psi_m$ collapse) and further apoptosis-related events [15,16,31]. From these contradictory data, it can be concluded that the involvement of mitochondrial permeability transition as an early step of apoptosis does not appear to be as universal as generally considered.

Yeast mitochondria do not have a typical PTP, but they support an unselective channel (yeast mitochondria unselective channel) which, although having characteristics distinct

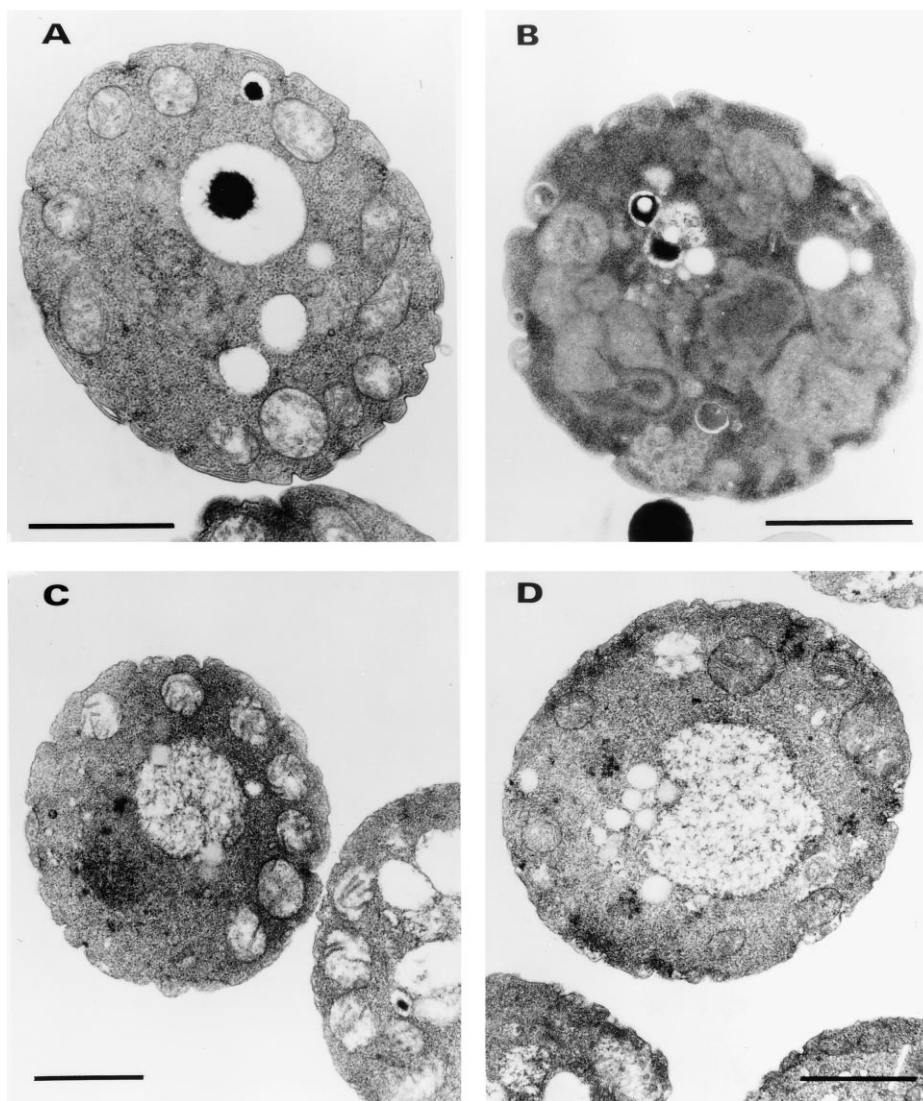


Fig. 4. Electron microscopy (EM) of *bax*-expressing yeasts. Cells were grown with lactate as a carbon source under non-expressing conditions and then transferred into the expression medium and spheroplasts were prepared and treated for EM (see Section 2). Typical images of individual cells are presented. (A) HT444-*bax* grown in the absence of galactose, (B) Ht444-*bax* after 3 h in the presence of 0.5% galactose, (C) WtB1 grown in the presence of doxycyclin, (D) WtB1 after 12 h in the absence of doxycyclin. The bar length represents 1 μ m.

from mammalian PTP, may have similar functions [32]. A way to evaluate the role of mitochondria permeability transition was to test the effect of *bax* in yeast strains inactivated for putative components of PTP. This has already been done with the outer membrane VDAC. VDAC-less cells are able to grow (although slowly) on a respiratory carbon source. The galactose promoter-driven expression of *bax* was successfully used with a clear result: VDAC is not involved in *bax*-effects [20].

Such an experiment was not possible with ANC-less strains, which are impaired for growth on respiratory carbon sources. An ANC-less strain carrying the tetracyclin promoter-driven *bax*-expression system was thus constructed (referred to as AtB1). The same test of lethality as for the wild-type strain, comparing glucose and mannose as carbon sources, gave a clear result: kinetics of *bax*-induced cell death are not significantly different for the ANC-less strain and for its wild-type (Fig. 3, middle).

3.4. *Bax-induced lethality in cytochrome c-less strains*

In yeast, there are two isoforms of cytochrome *c*, 1 and 2, encoded by *CYC1* and *CYC7* genes, respectively. Under aerobic conditions, isoform 1 represents about 95% of the total amount of the protein. The plasmid encoding *bax* under the control of the 'tetracyclin-off' promoter and carrying the auxotrophic marker *URA3* was introduced into the $\Delta CYC1::lacZ \Delta CYC7::CYH2$ strain B-06748.

The two tests already described for wild-type and ANC-less strains were performed. First, cells were grown in liquid glucose medium in the presence of doxycyclin to repress *bax*-expression. Cells were then harvested, washed and spread onto plates containing solid glucose medium without doxycyclin, that allows expression of *bax* (200 cells/plate). Results were identical for both wild-type and cytochrome *c*-less strains: cells were 100% killed following *bax*-expression (data not shown). This experiment demonstrates that cytochrome *c* is not required for *bax*-induced cell death.

As for the wild-type strain, the second test allowed us to estimate the kinetics of lethality following *bax*-expression. Cells were grown in a liquid medium that contained glucose or mannose as carbon source and in the presence of doxycyclin. After inoculation into a liquid medium without doxycyclin, an aliquot of the cultures was taken out at different time-points and spread on doxycyclin-containing plates. With glucose as a carbon source, kinetics of *bax*-induced cell death were the same for cytochrome *c*-less strains as for the wild-type (Fig. 3, bottom). With the non-repressive sugar mannose as a carbon source, kinetics of *bax*-induced cell death were strictly the same as with glucose (Fig. 3). This result differed markedly from results obtained on wild-type where kinetics of cell death with mannose (or other non-repressive sugars) were more rapid than with glucose. From data on wild-type, we had concluded that one or more glucose-repressed protein(s) were required for a fast *bax*-induced cell death. From data on a cytochrome *c*-negative strain, it can be concluded that cytochrome *c* is one of these proteins.

3.5. *Bcl-x_L*-rescuing effect in wild-type and cytochrome *c*-less strains

A plasmid encoding *bcl-x_L* under the control of the same 'tetracyclin-off' promoter and carrying an auxotrophic *TRP1* marker was introduced into the wild-type and cytochrome *c*-less strains already carrying the 'tetracyclin-off' plasmid encoding *bax*. Lethality tests were performed exactly as described above. For both tests, protection by *bcl-x_L* was total: 100% of the cells were able to grow when plated on a solid SC-glucose medium without doxycyclin (not shown). This was an important point to verify, since it shows that *bax*-induced cytochrome *c*-independent death is fully prevented by *bcl-x_L* co-expression.

3.6. *Bax*-induced cytochrome *c* release

We have reported previously that *GAL10*-driven *bax*-expression under respiratory conditions induced a relocalization of cytochrome *c* (and other proteins of the mitochondrial intermembrane space such as adenylate kinase) to the cytosol [20]. This was also observed in vitro with recombinant *bax* that was added to isolated yeast [20] or rat liver [17] mitochondria. Under both conditions, at least 50% of cytochrome *c* was relocalized from yeast mitochondria.

We therefore looked if the same phenomenon was observed with *tetO*-driven *bax*-expression. Mitochondria were isolated from cells grown in the presence of doxycyclin (control) or after different times of induction. Only a slight decrease of the amount of cytochrome *c* was observed both after a 6 or 18 h induction, in lactate or in glucose medium: whatever the conditions, at least 75% of cytochrome *c* remained associated to mitochondria, with an average value around 90% (as compared to $43 \pm 7\%$ with *GAL10*-driven *bax*-expression). This result is in accordance with the conclusion coming from the experiment on the cytochrome *c*-less strain, that massive cytochrome *c* relocalization is not absolutely required for *bax*-induced cell death.

3.7. Mitochondria morphology

The morphology of *bax*-expressing cells was assessed by electron microscopy on spheroplasts. Working on spheroplasts instead of intact cells allowed for a better view of the mitochondria structure in situ. Fig. 4 shows representative

cells observed in different spheroplast preparations. It is obvious that *GAL10*-driven expression of *bax* induces a dramatic alteration of cells. Mitochondria appear swollen and cristae cannot be distinguished. It should be noted that mitochondria isolated from such a preparation still support the permeability barrier of the inner-membrane but not the permeability barrier of the outer membrane [20]. On the opposite, mitochondria in spheroplasts prepared from *tetO*-driven *bax*-expressing cells do not exhibit any morphological difference compared to control cells. These results are perfectly consistent with data related to cytochrome *c* release.

3.8. Conclusions

A first important conclusion of this paper is that *bax*-effects in yeast differ depending on the type of metabolism. Under respiratory conditions (lactate), there is a strict correlation between *bax*-induced growth arrest and death. On the opposite, under fermentative conditions, although growth arrest occurs within a similar period of time as under respiratory conditions, *bax*-induced lethality is markedly delayed. This difference may be discussed in terms of the cellular ΔG_p . The intracellular ATP/ADP ratio of yeast may vary from two under strictly fermentative conditions to above 10 under respiratory conditions. Since we have already shown that ATP is required for an optimal effect of *bax* both in vivo and in vitro [20], the correlation between both observations seem obvious: a high ATP/ADP ratio is not necessary for *bax*-induced growth arrest but is required for a rapid *bax*-induced cell death. The second conclusion which can be drawn from these experiments comes from the difference in kinetics of *bax*-induced death in glucose as compared to non-repressive sugars. This allows us to conclude that at least one glucose-repressed protein causes the fast kinetics of death.

The second major conclusion of the present paper is that ANC is not required for *bax*-induced cell death, under these conditions. This is a critical point since neither VDAC [20] nor ANC (this paper), two major putative components of PTP, are required for *bax*-induced death and cytochrome *c* relocalization. In addition, we demonstrated previously that *bax*-induced cytochrome *c* relocalization occurs in yeast without any obvious permeability transition of the inner mitochondrial membrane [20]. Thus, this additional evidence argues against the involvement of any endogenous mitochondrial channel for *bax*-effects in yeast. This conclusion is somewhat contradictory with other reports where *GAL10*-driven *lexA-bax*-expression induced a growth arrest which was delayed in an ANC-less strain, as compared to a wild-type [25]. But it must be noted that, as shown in the present paper, *GAL10*-driven and *tetO*-driven *bax*-expression yield very different results concerning cytochrome *c* relocalization. Moreover, it has already been clearly shown that native *bax* has quite different effects in yeast from the *lexA-bax* fusion protein (compare results in [2] and [29,33]).

The third major conclusion is that cytochrome *c* is neither strictly required for *bax*-induced yeast cell death nor for the protective effect of *bcl-x_L*. However, since kinetics of *bax*-induced death of a cytochrome *c*-less strain are the same whatever the sugar (glucose or mannose), it can be inferred that cytochrome *c* is required for the faster *bax*-induced death as observed in wild-type cells grown in non-repressive sugars.

Galactose promoter and tetracyclin promoter-driven *bax*-expression systems may represent two different ways to under-

stand the molecular effects of *bax* in yeast. The first system gives a massive expression, which induced major alterations of mitochondrial structures ([2], this paper) (although the inner mitochondrial membrane remained intact considering its selective permeability [20]), massive cytochrome *c* relocalization [4,20] and rapid death [3]. The second system gives a lower expression, relatively slow death and conservation of the mitochondria structure. This expression system permits the confirmation of the role of cellular ATP in the kinetics of the death process. It also highlights a role for cytochrome *c*: it is not absolutely required for *bax*-induced death but its presence may improve the lethal action of *bax* in yeast.

Is it possible to understand any aspect of apoptosis form the yeast model? Recent hypotheses draw a functional relationship between cytochrome *c*-induced (or other apoptogenic mitochondrial proteins-induced) activation of caspases and a putative 'amplifying loop' where these activated proteases are thought to open PTP and cause an irreversible energetic collapse observed in most apoptotic cells. In this scheme, PTP-opening and further mitochondrial alterations should be a consequence, rather than a cause, of caspases activation. Mitochondria might be involved only in a secondary step linked to over-activation of apoptosis, but not in the primary apoptotic signal. Our conclusion, as reported above, underlines an amplifying role of cytochrome *c* for *bax*-effects in yeast and is in accordance with this hypothesis.

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