The antiapoptotic protein Bcl-x_L prevents the cytotoxic effect of Bax, but not Bax-induced formation of reactive oxygen species, in *Kluyveromyces lactis*

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The murine proapoptotic protein Bax was expressed in *Kluyveromyces lactis* to investigate its effect on cell survival and production of reactive oxygen species (ROS). Bax expression decreased the number of cells capable of growing and forming colonies, and it increased the number of cells producing ROS, as detected by both dihydrorhodamine 123 fluorescence and the intracellular content of SH groups. Mutation in the β -subunit of F₁-ATPase, or mitochondrial deficiency resulting from deletion of mtDNA (ρ^0 mutant), increased the sensitivity to Bax, indicating that Bax cytotoxicity does not require mitochondrial respiratory-chain functions. The antiapoptotic protein Bcl-x_L, when co-expressed with Bax, localized to the mitochondria and prevented Bax cytotoxicity. However, this co-expression did not prevent the production of ROS. These data suggest that in *K. lactis* cells expressing Bax, ROS are not the *sine qua non* of cell death and that the antiapoptotic function of Bcl-x_L is not limited to its antioxidant property.

Keywords: Bcl-2 proteins, oxygen radicals, *petite*-negative yeast, mitochondrial mutants

INTRODUCTION

Two yeast species, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, were used to demonstrate that characteristics of apoptotic cells, found in multicellular organisms, are present in unicellular eukaryotes (Sato *et al.*, 1994; Hanada *et al.*, 1995; Ink *et al.*, 1997). In *S. cerevisiae* the appearance of specific apoptotic markers, such as DNA breakage, chromatin condensation, loss of asymmetric distribution of plasma-membrane phosphatidylserine, and growth arrest preceding cell death, was observed after exposure to oxidative stress (Madeo *et al.*, 1999), in cells with a defective cell cycle (Madeo *et al.*, 1997) and in old mother cells (Laun *et al.*, 2001), as well as in cells expressing the proapoptotic members of the Bcl-2 family (Bax, Bak) (Ligr *et al.*, 1998; Madeo *et al.*, 1999). The onset of apoptosis induced by the latter two proteins was successfully prevented by co-expression of the antiapoptotic members of the Bcl-2 family (Bcl-2, Bcl-

x_L), suggesting that both types of mammalian proteins function in yeast similarly as in metazoan cells (for a review see Matsuyama *et al.*, 1999). Experimental data indicate that the cell-death-regulating activity of the Bcl-2 family members in mammalian cells depends on their ability to localize into mitochondrial membranes and to influence their functions. Bax expressed in yeast cells also localizes to mitochondria (Priault *et al.*, 1999; Gross *et al.*, 2000). Its expression induces mitochondrial dysfunctions (Manon *et al.*, 1997; Kiššova *et al.*, 2000), probably due to the formation of a channel in the outer mitochondrial membrane (Pavlov *et al.*, 2001). There are contradictory results in both mammalian and yeast cells as to what extent, if at all, the mitochondrial functions are required for apoptosis. It was shown, for instance, that human cells lacking mtDNA (ρ^0 cells) were resistant to *in vitro* induction of apoptosis by staurosporin (Dey & Moraes, 2000), suggesting that respiratory-chain function is required for apoptosis. Conversely, increased *in vivo* apoptosis in cells lacking mtDNA expression was recently observed (Wang *et al.*, 2001). Furthermore, *S. cerevisiae* mutants deficient in oxidative phosphorylation functions (Matsuyama *et al.*,

Abbreviations: DHR, dihydrorhodamine 123; PI, propidium iodide; ROS, reactive oxygen species; SM, synthetic medium.

1998; Marzo *et al.*, 1998; Harris *et al.*, 2000), or wild-type cells grown under fermentative conditions (Priault *et al.*, 1999), showed reduced Bax-induced toxicity and cell death (Gross *et al.*, 2000). On the other hand, it was demonstrated (Kiššová *et al.*, 2000) that the wild-type cells, as well as the mutants with defective oxidative phosphorylation due to mutations in either nuclear DNA, or mtDNA, needed 2–3 cell divisions in the presence of Bax before the growth arrest and cell death occurred.

An intriguing question in the cell-death pathway is the participation of reactive oxygen species (ROS) in the regulation of apoptosis. The observation that Bcl-x_L, the antiapoptotic member of the Bcl-2 family of proteins, has an apparent antioxidant function (Hockenbery *et al.*, 1993; Kane *et al.*, 1993) suggested a role of ROS in apoptosis. In some mammalian cells, antioxidants prevented the activation of caspases and cell death, indicating that ROS may act as signal molecules at the start of the death pathway (Maulik *et al.*, 1998; Tan *et al.*, 1998). On the other hand, ROS production could be a consequence of mitochondrial damage, or mitochondrial membrane hyperpolarization (Raha & Robinson, 2000; Aresenijevic *et al.*, 2000), both observed at the early stage of apoptosis in metazoa (Vander-Heiden *et al.*, 1997; Green & Reed, 1998). ROS were shown to accumulate in *S. cerevisiae* cells in which the apoptotic markers were induced by the defect in cell cycle regulation (*cdc48*^{S565G}) (Madeo *et al.*, 1997), Bax expression, oxidative stress (Madeo *et al.*, 1999), or cell ageing (Laun *et al.*, 2001). It was suggested that ROS might play a key role in the induction of apoptotic phenotypes and cell death in organisms lacking the major apoptosis regulators, members of the Bcl-2 family of proteins (Madeo *et al.*, 1999).

In the present work we investigated the effects of Bax and Bcl-x_L on ROS formation and cell death of *Kluyveromyces lactis*. In contrast to *S. cerevisiae*, which is a *petite*-positive, facultative anaerobic yeast, *K. lactis* is a *petite*-negative, strictly aerobic yeast in which respiration dominates over fermentation. Thus, the energy-yielding mechanism in *K. lactis* resembles that in mammalian cells, making it a better model for study of apoptosis. In addition, there are specific mutations in *MGI* genes encoding subunits of mitochondrial F₁-ATPase that convert *K. lactis* into *petite*-positive yeast (Chen & Clark-Walker, 1993; Clark-Walker *et al.*, 2000). Here we present evidence that the antiapoptotic protein Bcl-x_L localizes exclusively to the mitochondria, and prevents the cytotoxic effect of Bax, but does not eliminate the oxidative stress in Bax-expressing cells. We also show that in *K. lactis*, the cytotoxic effect of Bax, as well as the protective effect of Bcl-x_L, does not require functional mitochondrial oxidative phosphorylation.

METHODS

Yeast strains, plasmids and growth conditions. The parental wild-type *K. lactis* strain CK56-16C (α *ade1 lysA1 uraA1*) and the strain with a mutation in the β subunit of F₁-ATPase,

CK56-7A (α *ade1 lysA1 uraA1 mgi1-1*), were kindly provided by X. J. Chen (Australian National University, Canberra). Cells lacking mitochondrial DNA (ρ^0) were prepared by ethidium bromide mutagenesis from the *mgi1-1* mutant strain as described by Chen & Clark-Walker (1993).

A DNA fragment (*EcoRI*–*HindIII*) containing the murine Bax gene under the *GAL10* promoter from the YEp51-Bax plasmid (provided by J. C. Reed, The Burnham Institute, La Jolla, CA, USA) was cloned in centromere *K. lactis* shuttle plasmid pCXJ19 (provided by M. Wesolowski-Louvel, University of Lyon, France). A *HindIII* fragment containing the *URA3* gene from the YEp24 plasmid was further inserted into the *HindIII* site of the above construct. The murine Bcl-x_L gene was cloned under the *GAL1* promoter in the pCXJ19K vector. The latter was prepared by cloning the *PstI*–*SmaI* fragment containing the *LYS2* gene of *S. cerevisiae* from the YDp-K vector (Berben *et al.*, 1991). *K. lactis* cells were grown at 28 °C in complete medium containing 0.5% (w/v) Bacto yeast extract, 1% (w/v) Bacto-peptone, and 2% (w/v) glucose or 2% (w/v) galactose. Cells were transformed either by electroporation (Sanchez *et al.*, 1993) or using frozen competent cells (Dohmen *et al.*, 1991). The transformants were maintained in synthetic medium (SM: 0.67%, w/v, yeast nitrogen base) supplemented with indicated carbon source and nutritional requirements. To induce Bax and Bcl-x_L expression, the transformed cells grown to the early exponential phase in SM containing 2% glucose were washed and resuspended in SM containing 2% galactose. In the case of ρ^0 cells, the inducing medium contained 2% galactose plus 1% glucose.

Plating efficiency and viability assay. Fresh colonies of transformed *K. lactis* cells were resuspended in sterile water and equal numbers (300–500) of cells were plated in parallel on glucose- and galactose-containing SM plates. After 5–7 days the colonies were counted and the number of colonies on glucose plates was taken as 100%. The number of cells with compromised cell membrane permeability was determined after staining with 30 μ M propidium iodide (PI) using an Olympus BX-50 fluorescent microscope equipped with the corresponding filter.

Determination of intracellular ROS and SH groups. ROS were detected after incubating 10⁶–10⁷ cells in 500 μ l growth medium containing 50 μ M dihydrorhodamine 123 (DHR, Molecular Probes) for 15 min at room temperature. The samples were viewed with a fluorescent microscope equipped with a rhodamine optical filter.

SH groups in glutathione and soluble cellular proteins were assayed using 2,2-dithiobisnitrobenzoic acid (Hu *et al.*, 1994). Cells grown under inducing conditions were harvested, washed, resuspended in 0.25 M Tris/HCl, 0.02 M EDTA, pH 8.2, and broken by vortexing with glass beads. Homogenates were centrifuged at 4000 g for 5 min and the supernatant fractions were used to determine the SH groups and protein content. Total SH groups were calculated and normalized for protein content.

Western blots. Transformed cells grown under inducing conditions were harvested, washed and resuspended in 0.6 M sorbitol, 0.05 M EDTA, 0.05 M Tris/HCl, pH 7.5, supplemented with a cocktail of protein inhibitors (0.025 mM *N*- α -*p*-tosyl-L-lysine chloromethane, 0.5 mM *N*-tosyl-L-phenylalanine chloromethane, 1 mM phenylmethylsulfonyl fluoride, 5 mM aprotinin; Sigma). Cells were broken by vortexing with glass beads, the homogenates were centrifuged at 4000 g for 5 min and supernatants were collected. The supernatants were centrifuged at 10000 g for 10 min to

obtain a pellet fraction enriched in mitochondria and the supernatant fractions representing the cytosol. TCA-precipitated proteins from both the cytosol and mitochondrial fractions were solubilized in electrophoresis sample buffer and analysed by immunoblotting using Bcl-x_L (Santa Cruz Biotechnology) or ADP/ATP carrier antibodies (provided by I. Hapala, Institute of Animal Biochemistry and Genetics, Slovakia) and ECL-based detection.

Reproducibility of the results. All experiments were repeated at least three times. Data reporting plating efficiency and viability of cells are means values with standard deviations. The data for SH content are reported as a percentage of the SH value in cells transformed with control plasmids.

RESULTS

Expression of Bax and Bcl-x_L in *K. lactis*

Expression of mammalian Bcl-2 proteins in mitochondrial mutants of *S. cerevisiae* has resulted in a number of conflicting reports with regard to the mitochondrial functions required for their cytotoxic effect. We have shown that the reduced growth of cells with impaired mitochondrial functions on galactose is, at least in part, responsible for the reduced cytotoxicity of Bax in these cells (Kiššová *et al.*, 2000). To avoid the undesirable effect of carbon source on the growth of mitochondrial

Fig. 1. Induction of β -galactosidase activity in *K. lactis mgi1-1* and ρ^0 mutant cells. (a) The *mgi1-1* mutant strain [similar results were obtained with the parental (CK56-16C) strain (data not shown)] and (b) the corresponding mitochondrial (ρ^0) mutant, grown in SM with 2% glucose, were washed and transferred to fresh SM containing 2% glucose (■), 2% galactose plus 1% glucose (▲), or 2% galactose (●) as carbon source. Aliquots were withdrawn at the times indicated and the β -galactosidase activity was assayed in crude cell extracts as described by Guarente (1983).

Fig. 2. Plating efficiency of wild-type and mutant *K. lactis* cells expressing Bcl-2 proteins. Equal numbers of wild-type, *mgi1-1* and ρ^0 mutant cells transformed with two control plasmids (white bars), Bax-containing (black bars), or Bax and Bcl-x_L containing plasmids (hatched bars) were plated on solid SM containing 2% glucose or 2% galactose. In the case of the ρ^0 mutant the galactose medium was supplemented with 1% glucose. The numbers of colonies were scored after 7 days growth at 28 °C. The number of cells able to form colonies under inducing (galactose, or galactose plus glucose for ρ^0 cells) conditions is expressed as a percentage of the number of cells able to form colonies under non-inducing (glucose) conditions. The error bars represent standard deviations.

mutants, we expressed Bax and Bcl-x_L in *K. lactis*. An important advantage of this yeast is that the mitochondrial respiratory-deficient mutant (ρ^0) metabolizes galactose in the presence of glucose (Fig. 1). The evident lack of glucose repression thus allows an efficient induction of genes under the control of *GAL1–GAL10* promoters under respiratory-deficient conditions. This is not the case for *S. cerevisiae*, where most respiratory-deficient mutants grow poorly on galactose (Donnini *et al.*, 1992). As shown in Fig. 2, the wild-type and the mutant strains transformed with the control plasmids formed colonies with similar efficiency on both, glucose and galactose. Expression of Bax resulted in a reduction of plating efficiency of wild-type and *mgi1-1* mutant cells (to 60% and 20%, respectively). The strongest effect of Bax expression was observed in the mitochondrial respiratory-deficient (ρ^0) mutant. The plating efficiency of these cells was almost completely inhibited by Bax expression (Fig. 2). Similarly as in *S. cerevisiae* (Hanada *et al.*, 1995), co-expression of the antiapoptotic protein Bcl-x_L reversed Bax-induced growth arrest in all three *K. lactis* strains. To determine the viability of cells expressing Bax and Bcl-x_L, we stained the cells with PI (see Fig. 4 and Table 1). About 95% of the Bax-expressing respiratory-deficient (ρ^0) cells grown for 24 h in the inducing medium containing 2% galactose plus 1% glucose were stained with PI. The number of PI-stained cells was lower (62%) in the *mgi1-1* mutant as well as in the wild-type (see below, Table 1). Co-expression of Bcl-x_L prevented the Bax-induced PI staining in all three strains, suggesting that preventive effect of Bcl-x_L, like the toxic effect of Bax, did not require functional oxidative phosphorylation.

Table 1. Viability and ROS production, determined by PI and DHR staining, of cells expressing Bax and co-expressing Bax + Bcl-x_L after 24 h growth under inducing conditions

Strain	Stain	Percentage of PI- and DHR-stained cells transformed with plasmids:		
		Control	Control + Bax	Bax + Bcl-x _L
Wild-type	PI	5 ± 1.53	62 ± 2.03	8 ± 0.55
	DHR	9 ± 0.65	not given*	65 ± 1.67
<i>mgil-1</i>	PI	7 ± 0.68	62 ± 1.01	8 ± 2.11
	DHR	8 ± 1.05	not given*	59 ± 1.22
ρ^0	PI	3 ± 1.93	95 ± 1.06	5 ± 0.89
	DHR	4 ± 0.96	not given*	8 ± 1.65

* Data for DHR staining of Bax-expressing cells are not given due to non-specific staining of dead cells (see text).

Fig. 3. Western blots demonstrating that Bcl-x_L is localized in the mitochondrial fractions. Wild-type cells co-transformed with Bax- and Bcl-x_L-containing plasmids were grown in liquid SM with 2% galactose and at the times indicated aliquots of cells were removed, homogenized and fractioned to yield the cytosolic (C) and mitochondrial (M) fractions. The proteins of both fractions were analysed by Western blotting using (a) Bcl-x_L antibody and (b) antibody against mitochondrial ADP/ATP carrier (AAC).

Bcl-x_L protein localizes to the mitochondria of *K. lactis*

It has recently been shown that Bax protein expressed in *S. cerevisiae* is localized in mitochondria (Priault *et al.*, 1999; Gross *et al.*, 2000) and influences the mitochondrial metabolism (Manon *et al.*, 1997; Kiššová *et al.*, 2000). We examined whether Bcl-x_L was localized in the same organelles. The Western-immunoblotting analysis of crude mitochondrial and cytosol fractions of Bcl-x_L-expressing *K. lactis* cells demonstrated that Bcl-x_L protein was localized in the mitochondrial fraction, as soon as 6 h after induction in galactose medium and was still present there 48 h after induction (Fig. 3).

Expression of Bax leads to accumulation of ROS and a decrease of intracellular SH content

To follow Bax-induced intracellular accumulation of ROS, we stained the Bax-transformed cells with DHR, which is oxidized to the fluorescent rhodamine 123 by

peroxides. As shown in Fig. 4(a, b) and Table 1, most of the wild-type and ATPase (*mgil-1*) mutant cells simultaneously expressing Bax and Bcl-x_L were stained with DHR, but not with PI, indicating that Bcl-x_L suppressed cell death though it did not suppress ROS formation in these cells. DHR also stained similar proportions of Bax-expressing wild-type and ATPase (*mgil-1*) mutant cells (not shown), but it should be taken into account that dead cells became non-specifically stained with DHR and contributed to the apparent numbers of DHR-positive cells. Nevertheless, the percentages of positive cells in this case were still no greater than with cells expressing Bax + Bcl-x_L, consistent with Bcl-x_L not eliminating the oxidative stress in these cells. In contrast to the above two respiratory-competent strains, the respiratory-deficient (ρ^0) cells protected from Bax-induced death by expression of Bcl-x_L were scarcely stained by either PI or DHR (Fig. 4c, Table 1).

To determine whether antioxidants can have a protective effect on Bax-triggered inhibition of growth and viability of *K. lactis* cells, glutathione was included in the induction medium. Within the concentration range 1–25 mM glutathione we did not observe a protective effect either on the plating efficiency or on the viability of Bax-expressing wild-type and mutant cells (data not shown).

The observed effects of Bcl-2 proteins on ROS formation in *K. lactis* cells were further confirmed by assessing another indicator of oxidative stress, the intracellular pool of SH groups. As shown in Fig. 5(a), H₂O₂ and antimycin A, which are well-known inducers of ROS formation in many different cell types, decreased the intracellular SH content in *K. lactis* to different extents depending on the time of exposure. Bax expression in wild-type and ATPase (*mgil-1*) mutant cells had a similar effect to H₂O₂ and antimycin A, resulting in a decrease of the intracellular pool of SH groups as compared to the cells transformed with control plasmids (Fig. 5b). In agreement with the results obtained by

Fig. 4. ROS accumulation in *K. lactis* cells expressing Bcl-2 proteins. Wild-type (a), *mgi1-1* (b) and ρ^0 (c) cells were grown under inducing conditions for 6 h. Cells transformed with control plasmids (1), Bax- and Bcl- x_L -containing plasmids (2), or control and Bax-containing plasmids (3) were grown under inducing conditions. Aliquots were withdrawn, stained with either DHR or PI, and analysed by fluorescence microscopy

Fig. 5. Oxidative stress and Bax expression affect the intracellular content of SH groups. (a) Wild-type cells grown in SM with 2% galactose were treated with either 5 mM H₂O₂ (white bars) or 7.5 μ M antimycin A (black bars). At the indicated times aliquots of cells were removed, homogenized, and the content of SH groups was determined. The intracellular SH contents are shown as a percentage of the SH content of untreated control cells. (b) Cells of the indicated genotypes co-transformed either with control plus Bax-encoding plasmids (white bars), or with Bax- plus Bcl- x_L -encoding plasmids (black bars) were grown to the exponential phase in liquid SM containing glucose. The cells were then transferred to galactose-containing medium and the intracellular SH content was determined after 6 h. The values are reported as a percentage of the SH content in cells growing under non-inducing conditions. [The absolute values for SH content corresponding to 100% were: 0.28 ± 0.008 mmol (mg protein)⁻¹ for wild-type, 0.20 ± 0.009 mmol (mg protein)⁻¹ for the *mgi1-1* strain and 0.18 ± 0.004 mmol (mg protein)⁻¹ for the ρ^0 strain]. Error bars represent standard deviations.

staining, the oxidative stress induced by Bax was stronger in the *mgi1-1* mutant than in the wild-type, and more importantly, it was not suppressed by Bcl- x_L . The cellular SH content in the mitochondrial respiratory-deficient ρ^0 mutant remained unchanged after expression of Bax alone, or of Bax and Bcl- x_L together (Fig. 5b). It should be noted that the expression of Bcl- x_L alone had no effect either on DHR fluorescence, or on the intracellular SH content, in any of the tested strains (data not shown).

using an appropriate filter. Data for DHR staining of Bax-expressing cells are not shown due to the non-specific staining of dead cells. VIS, examined under visible light.

DISCUSSION

In this work we investigated the response of *K. lactis* cells to two proteins, Bax and Bcl-x_L, that are regulators of apoptosis in metazoa. *K. lactis* possess several advantages for investigation of the role of Bcl-2 proteins in apoptosis. Like most mammalian cells, *K. lactis* are strictly aerobic, with an intensive mitochondrial oxidative metabolism. There are mutant strains of *K. lactis* (Clark-Walker *et al.*, 2000), including the *mg1-1* mutant used in this work, in which the mtDNA can be deleted to produce mitochondrial respiratory-deficient (ρ^0) mutants. It is also important that the low glucose repression in the ρ^0 mutant (Fig. 1) allowed the cells to metabolize galactose in the presence of glucose; therefore *GAL* promoters can be efficiently used for gene expression under respiratory-deficient conditions.

We expressed the murine Bax and Bcl-x_L genes in the parental wild-type *K. lactis* strain, in the *mg1-1* mutant with a defect in the β subunit of F₁-ATPase, and in the corresponding ρ^0 mutant, and measured the growth and viability of transformed cells. As demonstrated by plating and PI exclusion assays, *K. lactis* cells responded to Bax expression by growth arrest and cell death, similar to the response in *S. cerevisiae*. Bcl-x_L, when co-expressed with Bax, localizes in mitochondria and almost completely prevents the cytotoxic effect of Bax in *K. lactis*. In contrast to results pointing to reduced Bax cytotoxicity in cells with nonfunctional mitochondrial oxidative phosphorylation (Harris *et al.*, 2000; Gross *et al.*, 2000; Matsuyama *et al.*, 1998; Priault *et al.*, 1999), the mitochondrial mutants of *K. lactis* were even more sensitive to Bax than the corresponding wild-type strain. Therefore, our results suggest that the cytotoxic effect of Bax, as well as the preventive action of Bcl-x_L, do not require functional mitochondrial oxidative phosphorylation. One possible explanation for the discrepancy between *S. cerevisiae* and *K. lactis* could relate to the fact that the mitochondrial mutants of *S. cerevisiae* have a reduced ability to metabolize and to grow on galactose (Donnini *et al.*, 1992; Kiššová *et al.*, 2000), whereas *K. lactis* mitochondrial mutants do not grow on galactose at all. As mentioned above, *K. lactis* ρ^0 cells lacked glucose repression, as evidenced by the ability of galactose to induce β -galactosidase activity in the presence of glucose. If we take into account that Bax protein is targeted to the yeast mitochondria (Priault *et al.*, 1999; Gross *et al.*, 2000), the high sensitivity of *K. lactis* ρ^0 mutant to Bax could be due to an additive mitotoxic effect (Skulachev *et al.*, 2000) in this 'petite-negative' species. The higher Bax cytotoxicity in ρ^0 cells as compared to the corresponding parental strain might be also attributed to the presence of glucose in the inducing medium. However, the undisturbed growth of ρ^0 cells transformed with either control or Bax + Bcl-x_L-containing plasmids indicates that this is not the case.

As seen in mammalian cells, Bcl-x_L, when expressed in *K. lactis*, was largely localized to the mitochondria and efficiently prevented Bax-induced growth arrest and PI staining. However, Bcl-x_L did not suppress ROS forma-

tion in these cells, indicating that its protective effect does not necessarily include an antioxidant action, or activation of antioxidant defence. Bcl-x_L also protected ρ^0 cells from Bax-induced growth arrest and death. However, in contrast to the wild-type only a few ρ^0 cells simultaneously expressing Bax and Bcl-x_L were stained by DHR. In addition, the intracellular SH contents of ρ^0 cells did not show any significant change after Bax and/or Bcl-x_L expression. Although a limited ROS generation in mammalian ρ^0 cells was reported (Jacobson *et al.*, 1993; Shimizu *et al.*, 1995), so far we have no explanation for the observation that the *K. lactis* ρ^0 cells, regarding ROS formation and the cellular SH content, responded differently from the respiratory-competent cells as regards the expression of Bcl-x_L.

ROS are considered to be a potent trigger of apoptosis in some experimental systems (Liu *et al.*, 1996; Maulik *et al.*, 1998; Tan *et al.*, 1998) and it was suggested that Bcl-x_L plays an antioxidative role, possibly by protecting the cellular constituents from oxidative damage (Hockenbery *et al.*, 1993; Longo *et al.*, 1997). The apoptotic phenotypes of *S. cerevisiae* induced by different factors, including Bax, are also accompanied by formation of ROS within the cells (Madeo *et al.*, 1999; Laun *et al.*, 2001). Our finding that the cytotoxic effect of Bax in *K. lactis*, but not Bax-induced ROS formation, is inhibited by Bcl-x_L indicates that the ROS are not in the main pathway of Bax-induced death of *K. lactis* cells and that the antiapoptotic effect of Bcl-x_L protein includes, besides the antioxidative properties, also other mitochondrially related mechanism(s).

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