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A

CLONING AND PARTIAL CHARACTERIZATION OF THE NOVEL *BLM3* GENE

by

DONNA EVANS FEBRES

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2001

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ABSTRACT

CLONING AND PARTIAL CHARACTERIZATION OF THE NOVEL *BLM3* GENE

by

Donna Evans Febres

Advisor: Professor Carol Wood Moore.

Mutational alteration of the *BLM3* gene in *Saccharomyces cerevisiae* confers hypersensitivity to lethal effects of ionizing radiation, bleomycin and structurally-related phleomycin. Bleomycin is an anticancer drug used clinically in the treatment of many types of cancers. In the absence of bleomycin or structurally-related phleomycin, the *blm3-1* mutant cells were smaller in size, grew more slowly than *BLM3* cells, and displayed decreased viability. Preliminary studies showed that the viability of mutant cells was enhanced after exposure to 2 M cations (Na^+, K^+) and 0.005% sodium dodecyl sulfate (SDS), while the viability of *BLM3* cells decreased after these exposures. The enhanced viability of mutant cells suggested an alteration in membrane integrity. The *BLM3* gene was cloned by functional complementation of the phleomycin hypersensitivity conferred by the *blm3-1* mutation. The nucleotide sequence of *BLM3* encodes a predicted integral protein of 1804 amino acids with seven to ten potential transmembrane domains and additional motifs. The null mutant, *blm3Δ ::HIS3*, was created by gene replacement. In the absence of the bleomycin-phleomycin group of antibiotics, the *BLM3* gene is not essential for viability. Mutant *blm3Δ::HIS3* cells in the absence of bleomycin and

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structurally-related phleomycin were not significantly smaller in size, grew only slightly slower than related *BLM3* cells, and did not display decreased viability. Preliminary studies showed that the viability of mutant *blm3Δ ::HIS3* cells was not enhanced after exposure to 2 M cations (Na^+, K^+) and 0.005% SDS compared to the viability of related *BLM3* cells. Based on preliminary localization studies, Blm3p appeared to be localized around the cell surface. Based on all of these studies, we propose that Blm3p may play a role in structural integrity of the plasma membrane, cellular growth, and membrane permeability. The possible role of Blm3p as a transporter is also discussed.

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Dedicated to my family and in loving memory of my father
Thomas J. Evans

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TABLE OF CONTENTS: **PAGE**

Introduction:

I. ***Saccharomyces cerevisiae*** **as a model organism** 1
- A. Radiation effects 1
- B. The cell cycle and DNA repair 2

II. **Bleomycins and structurally related phleomycin** 3
- A. Structure and mechanism of action 3
- B. Cell Membranes 4

III. **Control of cellular cytotoxcity of bleomycin molecules** 4
- A. Entry and accumulation of bleomycin into animal and human cells 4
- B. Bleomycin hydrolase 5
- C. DNA repair pathways induced by bleomycin damage 5
- D. Bleomycin transporters 6

IV. **Plasma membrane: composition and integrity** 6
- A. Lipids 7
- B. Proteins 7

V. **The** ***blm3-1*** **mutation of** ***Saccharomyces cerevisiae*** 8

VI. **Major findings** 9

Materials and Methods: 10

1. Strains and plasmids 10
2. Media and growth conditions 10
3. Yeast transformation, *E. coli* transformation and plasmid isolation 11
4. Cloning of the gene by complementation 12
5. DNA sequencing 12
6. Computer analysis 12
7. *SMC1* screening 13
8. Construction of the gene deletion 13

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9. Construction of the *BLM3*-GFP fusion 15

10. Microscopy 17

11. Mitotic growth and cell size assay 17

12. Assay for respiration deficiency 18

13. Viability Assay 18

14. Cation treatments 18

15. SDS treatments 19

16. Calcofluor treatments 19

17. Northern blot analysis 19

Results: 19

Part 1: Cloning by complementation 19
1. Cloning of the *BLM3* gene 19

Part 2. Computer analyses 20
1. Computer analyses of *BLM3* 20
2. *SMC1* screening 21
3. Predicted amino acid sequence of Blm3p 22
4. Comparison of Blm3p to other proteins characterized as having transport functions 22

Part 3. Partial characterization of *BLM3* 23
1. Localization of Blm3p 23

2. Construction of the *BLM3* null 24

3. Viability and drug resistance of *BLM3* null mutants 25

4. Cell Wall Assay 26

5. Northern blot analyses 27

6. Additional phenotypic analyses 27
a. Colony and cell size difference in ***blm3-1*** cells compared to *BLM3* cells 27
b. Assay for respiration deficiency 28
c. Mitotic growth 28

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7. Membrane Integrity Studies 29
a. Cation induced death 30
b. SDS membrane alteration assay 30

Discussion: 31

1. Blm3p is a novel membrane protein 31

2. The *blm3-1* and *blm3-1Δ ::HIS3* mutant strains are hypersensitive to killing by bleomycin and phleomycin 32

3. Membrane integrity and phenotypes of *blm3-1* cells 34

4. The *blm3-1* mutant cells are smaller than *BLM3* 35

5. Smaller colony sizes of *blm3-1* cells is not due to respiration deficiency 36

6. Different phenotypes exhibited in the *blm3-1* and *blm3-1Δ ::HIS3* strains 36

7. While computer aided analyses suggest Blm3p is a permease, its role as a transporter remains to be determined 37
a. The possible role of Blm3p as an antiporter 37
b. Possible role of Blm3p as an MDR protein 40

References: 76

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List of Tables:

Materials and Methods.

Table 1. Strains and plasmids used 42
Table 2. Primers for replacing *BLM3* gene with *HIS* 44
Table 3. Primers for fusing GFP to the 3' end of *BLM3* 45

Results.

Table 3-1. Co- segregation of *HIS3* marker and phleomycin hypersensitivity 46

Table 3-2. Growth of *BLM3* and mutant *blm3-1* and *blm3Δ ::HIS3* strains on SC + Calofluor 47

Table 3-3. Expression or lack of expression of the 5.4 kb *BLM3* transcipt in human tissues 48

Table 3-4. Liquid Assays for growth with and without 0.005% SDS 49

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List of Figures:

INTRODUCTION.

Figure 1. Phases in the life cycle of a yeast cell 50

Figure 2. Structures of bleomycins and phleomycins 51

Figure 3. Survival of *BLM3* (CM1313-1C) cells and *blm3-1* mutant (CM1313-1B) cells 52

Figure 4. Deletion of the chromosomal *BLM3* gene. 53

RESULTS.

Part 1.

Figure 1-1. Multicopy plasmid shuttle vector, YEp24 and restriction map 54

Figure 1-2. Complementation of the *blm3-1* mutation 55

Part 2.

Figure 2-1. Region of chromosome VI which has been studied 56

Figure 2-2. The *SMC1* gene does not complement the *blm3-1* mutation 57

Figure 2-3. *BLM3* nucleotide sequence, the predicted amino acid sequence and hydropathy plot 58

Part 3.

Figure 3-1. PCR verification of the cassette containing GFP (S65T mutant version of GFP) and the selectable marker *HIS3* 63

Figure 3-2. Blm3p-GFP phleomycin resistant transformants 64

Figure 3-3 Verification of the correct GFP integration fused to the 3'end of *BLM3* 65

Figure 3-4. Confocal micrograph of Blm3p-GFP 66

Figure 3-5. PCR verification of the *HIS3* disruption cassette in the pRS303 plasmid 67

Figure 3-6. Analytical PCR verification for the disruption of *BLM3* 68

Figure 3-7. Tetrad analysis of the *BMA1* diploid strain transformed with the disruption cassette corresponding to *BLM3* 69

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Figure 3-8. Light micrographs exhibiting cell sizes of A) *BLM3* (CM1469-5A) and B) *blm3-1* (CM1469-2A), 40x magnification C) *BLM3* (CM1522-9A) and D) *blm3Δ ::HIS3* (CM1522-9B), 20x magnification. 70

Figure 3-9. Reduced viability for *blm3-1* compared to *BLM3* 71

Figure 3-10. Comparisons of growth curves of *BLM3* (CM1469-5A) and. *blm3-1* (CM1469-5C) strains (A) and *BLM3* (CM1522-9A) and *blm3-Δ ::HIS3* (CM1522-9B) strains (B) 72

Figure 3-11. Strain viability of *BLM3* and *blm3-1* strains after exposures to cationic solutions (NaCl, KCl, $MgCl_2$, $CaCl_2$). 73

Figure 3-12. Strain viability of *BLM3* and *blm3Δ ::HIS3* strains after exposures to cationic solutions (NaCl, KCl, $MgCl_2$, $CaCl_2$). 74

Figure 3-13. Growth of *BLM3* and *blm3-1* cells with and without SDS (0.005%) 75

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INTRODUCTION

I. *Saccharomyces cerevisiae* as a model organism.

Saccharomyces cerevisiae is a frequently used model for studying the effects of oxidative damage on biological systems. There are many advantages for using the single-celled eukaryote, *S. cerevisiae*. Understanding its responses to oxidative damage, including radiation, may help interpret the effects of such damage on higher eukaryotes, including man. The properties that make yeast suitable for biological studies include its ability to grow rapidly, a well-defined genetic system, a simple life cycle, being able to exist as a stable haploid or diploid or even in a state of higher ploidy, and the capability of being transformed in gene-cloning studies.

A. Radiation effects. In *S. cerevisiae*, bleomycin is said to be "radiomimetic" in that it produces biological effects similar to those observed after exposure of cellular DNA to ionizing radiation (Umezawa *et al.*, 1966; Povirk 1983; Moore, 1978, 1982a,b, 1991). The main targets for radiation damage in yeast are DNA (Zelle and Hollaender, 1955; Bashkirov *et al.*, 2000; Brennan and Schiestl, 2001) and cellular membranes (Myers, 1970; Khare *et al.*, 1982; Ferlini *et al.*, 1999; Benderitter *et al.*, 2000). X-ray induced death in human cells shows an alteration in the plasma membrane that involves changes in membrane permeability and membrane potential (Ferlini *et al.*, 1999). Benderitter and coworkers (2000) have shown that x-rays produce a loss in membrane integrity that results in changes in membrane fluidity and cell death.

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The existence of DNA repair mechanisms became apparent with the discovery that visible light as a post-irradiation treatment repairs DNA damage following exposure to ultraviolet radiation (Zelle and Hollaender, 1955).

There are three major double-strand break repair pathways identified in *S. cerevisiae* (reviewed by Moore *et al.*, 2000) They include, *RAD52*-dependent homologous recombination repair, Ku-protein dependent complementary end-joining or nonhomologous repair and single-strand annealing or nonconservative pathway in direct-repeat DNA (reviewed by Moore *et al.*, 2000; Xiao *et al.*, 2000).

Many radiation-sensitive mutants of *S. cerevisiae* are referred to as *rad* mutants (Game and Mortimer, 1974). The *rad* mutants have been placed into the three epistatic groups *RAD3*, *RAD52* and *RAD6*. The proteins encoded by the genes in the *RAD3* group are involved in excising ultraviolet-induced lesions from DNA and responsible for filling gaps left by the excised lesions and adjoining nucleotides using the opposite intact strand as the template. The proteins encoded by genes in the *RAD52* group mainly repair DNA double-strand breaks by recombination. The *RAD6* group is made up of an error-prone mutagenesis pathway and two error-free postreplication repair pathways (Xiao *et al.*, 2000). The genes in the *RAD6* group are induced by UV and sporulation (Prakash, 1989; Xiao *et al.*, 2000).

B. The cell cycle and DNA repair. The yeast cell cycle involves growth and the subsequent division of cells. It is divided into a nuclear division also known as mitosis (M phase), a G1 gap period before a DNA synthesis (S phase), and a post-synthetic G2 phase (**Figure 1**) (Howard and Pelc, 1953). The cell cycle is transiently arrested at G1/S, intra S and G2/M stages (Longese *et al.*, 1998). These positions are

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known as DNA damage checkpoints (Hartwell and Kastan, 1994) that establish a link between DNA repair and cell cycle progression (**Figure 1**). Checkpoints can be referred to as intrinsic and extrinsic mechanisms (Longhese *et al.*, 1998). Intrinsic mechanisms occur in each cell cycle to ensure the proper order of events under normal conditions. The extrinsic mechanisms are activated when DNA damage is detected. The latter mechanism activates genes in pathways involved in repairing damaged DNA.

II. Bleomycins and Structurally Related Phleomycins:

A. Structure and mechanism of action. The bleomycins comprise a family of hydrophilic antitumor antibiotics, isolated from the bacterium Streptomyces verticillus (Umezawa *et al.*, 1966; Ishizuka *et al.*, 1967). The bleomycins are oxidative DNA damaging agents used clinically for the treatment of malignancies of the head and neck, squamous cell carcinomas, testicular cancer and Hodgkin's and nonHodgkin's lymphomas (Blum *et al.*, 1973; Mir *et al.*, 1996). Bleomycin is used as a chemotherapeutic agent because of its ability to introduce double-strand breaks in DNA (Umezawa *et al.*, 1966, 1972).

The bleomycins consist of approximately 200 structurally related molecules that differ from each other at their terminal amine moiety (Umezawa *et al.*, 1966, 1979; Mir *et al.*, 1996). The commercial product used in clinical cancer treatments, Blenoxane, consists of a mixture of eleven of the bleomycin molecules, predominantly bleomycin-A_2 and bleomycin-B_2 (**Figure 2**). Phleomycins are structurally related to bleomycins (Umezawa, 1979), and differ from bleomycins in

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the oxidation state of their sulfur heterocycles (**Figure 2**). In this study, phleomycin was used in the cloning and partial characterization of the *BLM3* structural gene.

Functionally, the bleomycin molecule can be divided into two domains, a metal-binding domain and a DNA-binding domain that includes the terminal amine and bithiazole group (**Figure 2**). The metal-binding domain can bind metals such as Fe^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+} and Cu^{2+}. Bleomycin in the presence of oxygen and Fe(II) cleaves DNA through a method involving the formation of free radicals (Petering *et al.*, 1990; Steighner and Povirk, 1990, reviewed by Burger, 1998).

B. Cell Membranes. Bleomycin has also been shown to cause damage to the plasma membrane in *S. cerevisiae* (Moore *et al.*, 1992). It does so by creating lesions in the cell wall that extend into the cell membrane (Moore *et al.*, 1992; Beaudouin *et al.*, 1993). Bleomycin also damages human cell membranes (Moore *et al.*, 1985).

III. Control of cellular cytotoxicity of bleomycin molecules.

Bleomycin cytotoxicity appears to be controlled by four major mechanisms in intact cells. These mechanisms include drug accumulation within the cell (Mir *et al.*, 1996), the action of bleomycin hydrolase (a cysteine protease) that detoxifies bleomycin (Akiyama *et al.*, 1981), cellular DNA repair mechanisms (Mir *et al.*, 1996), and bleomycin transporters (Long *et al.*, 1988; Glazebrook *et al.*, 1993; LeVier *et al.*, 2000). Membrane alteration has also been proposed as a bleomycin resistance mechanism (Pron *et al.*, 1994).

A. Entry and accumulation of bleomycin into animal and human cells. The entry of bleomycin into the cell seems to involve the plasma membrane. Bleomycin, however, is unable to diffuse through the plasma membrane in animal

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cells. Bleomycin is recognized at the cell surface of both Chinese hamster fibroblasts and human cells by an unidentified membrane-bound protein believed to be involved in the internalization and cytotoxicity of the molecule (Pron *et al.*, 1993, 1994 and 1999). The existence of bleomycin binding sites has been associated with bleomycin cytotoxicity in Chinese hamster fibroblasts (Pron *et al.*, 1994) and human cells (Pron *et al.*, 1999). A decrease in the number of bleomycin binding sites has been observed in bleomycin resistant cells compared to bleomycin sensitive cells. Pron and co-workers (1999) have proposed a mechanism of receptor-mediated internalization of bleomycin in human cells. The membrane protein that specifically binds bleomycin is considered to be a receptor protein. Pron and co-workers (1999) hypothesized that following the binding of cytotoxic bleomycin molecules to this potential membrane receptor, bleomycin molecules are internalized by plasma membrane engulfment while the cell is undergoing endocytotic activity.

B. Bleomycin hydrolase. Bleomycin hydrolase is found in both mammalian (Sebti *et al.*, 1989) and yeast cells (Kambouris *et al.*, 1992). Cells possessing high levels of bleomycin hydrolase are able to withstand exposure to relatively high doses of bleomycin compared to cells possessing low levels of the enzyme (Jani *et al.*, 1992 and Morris *et al.*, 1992). Bleomycin hydrolase is believed to be located in the cytosol where it is able to degrade bleomycin molecules, resulting in a decrease in bleomycin cytotoxicity (Pron *et al.*, 1999).

C. DNA repair pathways induced by bleomycin damage. Bleomycin damage has been shown to induce error-prone repair in yeast (Severgnini *et al.*,

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1991), SOS repair in *E. coli* (Povirk *et al.*, 1988) and long patch excision repair in human fibroblasts (DiGiuseppe and Dresler, 1989). Recombinational repair, base-excision repair and post-replication repair are the most important repair mechanisms for bleomycin induced DNA damage (reviewed by Moore, 1999). Since bleomycin-induced DNA damage is considered to be radiomimetic, repair pathways of chromosomal damage by bleomycin and ionizing radiation are shared.

D. Bleomycin Transporters. According to Ichige and Walter (1997), alterations in the plasma membrane may be associated with bleomycin resistance in bleomycin transporters. The bleomycin transporters encoded by *SbmA* in *E. coli* and by *BacA* in *Rhizobium meliloti* are involved in bleomycin resistance (Long *et al.*, 1988; Glazebrook *et al.*, 1993; LeVier *et al.*, 2000). The *BacA* gene may also be important in the maintenance of membrane integrity (Ichige and Walker, 1997). It was suggested that the increased resistance to bleomycin conferred by *bacA* is due to an alteration in the membrane integrity that causes reduced uptake of bleomycin (Ichige and Walker, 1997). Although the exact alteration in the membrane structure is unknown, Ichige and Walker (1997) suggest the membrane defect might cause the *bacA* mutants to become more sensitive than wild-type strains to environmental conditions, such as ethanol.

IV. Plasma membrane: composition and integrity.

The yeast plasma membrane has been shown to be essential for cell viability, normal growth rates, maintenance of structural integrity, membrane-bound enzymes and membrane permeability (Molzahan and Woods, 1972;

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Bottema *et al.*, 1983; Mager and Varela, 1993; Skaggs *et al.*, 1996). The plasma membrane in all prokaryotic or eukaryotic cells acts as a barrier separating the aqueous interior of the cell from its aqueous exterior. This barrier is a unit membrane that forms the cell and is made up of lipids and proteins.

A. Lipids. Like most biological membranes, yeast plasma membranes contain only amphipathic lipids. Yeast plasma membranes contain mainly phospholipids (glycerophospholipids) and sterols (Henschke and Rose, 1987; van der Rest *et al.*, 1995). The neutral lipids, triacylglycerols and sterol esters have been detected in some yeast plasma membrane preparations. Phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol and phosphatidylserine are the principal lipids found in yeast plasma membranes (Henschke and Rose, 1987). Phosphatidylglycerol has also been found in yeast plasma membranes in small amounts (Henschke and Rose, 1987). Ergosterol is the major sterol found in *S. cerevisiae* and other yeast species (Brevik and Owades, 1957; Demel and De Kruyff, 1976).

B. Proteins. Proteins in the plasma membrane have many functions in yeast. Some of these proteins function as transporters involved in the transport of simple sugars, oligosaccharides, inositols, drugs, amino acids, nucleosides, organophosphate esters, Krebs cycle metabolites, and a large variety of organic and inorganic anions and cations (Pao *et al.*, 1998). Others function as ATPases to provide a proton-motive force (PMF) across the membrane (van der Rest *et al.*, 1995), enzymes catalyzing reactions involved in the synthesis of cell wall

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components, and receptors which include G protein receptors involved in signal transduction (Henschke and Rose, 1987).

Movement of ions across permeases in the plasma membrane is caused by a difference in the electrical charge or differences in the concentration of ions between one side of the membrane and the other. The membrane potential that results is due to a difference in the number of positive and negative charges across the membrane established by the positively charged cations and the negatively charged anions existing on each side of the membrane.

V. The *blm3-1* mutation of *Saccharomyces cerevisiae.*

To increase our understanding of oxidative damage and of the mechanism of action of the bleomycin-phleomycin family of antibiotics on eukaryotic cells, several *S. cerevisiae* mutants were isolated on the basis of their hypersensitivities to killing by bleomycins and phleomycins (Moore, 1980, 1991). Interestingly, one mutation, codominant *blm3-1* (**Figure 3**), fell into a unique genetic complementation group (Moore, 1991) and was chosen to be studied. This thesis reports the cloning and partial characterization of the *BLM3* gene.

Many of the *blm* mutants displayed cross-sensitivities to killing by radiation and hydrogen peroxide (Moore, 1980, 1991). The cross sensitivities of the *blm* mutant strains to ionizing radiation and radiomimetic bleomycin suggested that these mutants are likely to be altered in processes acting directly or indirectly on DNA damage. However, *blm3-1* mutant strains in comparison to the other *blm* mutants were only slightly hypersensitive to x-rays and hydrogen peroxide. Later studies showed that radiomimetic bleomycin also caused damage

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to the plasma membrane in *S. cerevisiae* by creating lesions in the cell wall that extend into the cell membrane (Moore *et al.*, 1992; Beaudouin *et al.*, 1992). Since *blm3-1* mutant cells were only slightly hypersensitive to x-rays and hydrogen peroxide and bleomycin damages plasma membranes and cell walls, a working hypothesis for this project was that the *BLM3* gene could encode a membrane or cell wall protein.

VI. Major findings.

The *BLM3* gene was cloned by functional complementation of the phleomycin hypersensitivity conferred by the *blm3-1* mutation. The nucleotide sequence of *BLM3* encodes a predicted integral protein of 1804 amino acids with seven to ten potential transmembrane and additional motifs. It is a non-essential gene for growth in the absence of bleomycin and structurally-related phleomycin. Computer analyses suggested the Blm3p could be a potential member of the major facilitator superfamily (MFS) of permeases (Nelissen *et al*, 1997). Blm3p appears to localize around the cell periphery according to GFP localization studies.

Compared to *BLM3* cells, *blm3-1* mutant cells are hypersensitive to killing by bleomycin and phleomycin, display decreased cellular viability, are smaller in size, grow at a slower rate, and exhibit increased viability in the presence of high concentrations of cations $Na^+$and K^+ (2 M) and SDS (0.005%). Compared to *BLM3* cells, the *blm3-Δ::HIS3* mutant cells are hypersensitive to killing by phleomycin, but to a lesser degree than *blm3-1* cells, do not grow significantly slower, do not display decreased cellular viability, and do not display increased viability in the presence of high concentrations of cations $Na^+$and K^+ (2 M) and SDS (0.005%). Thus, *blm3-1*

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mutant cells appear to be more resistant than *BLM3* cells to harsh environmental conditions such as cations Na^+ and K^+ and the plasma membrane destabilizer SDS, possibly due to some alteration in the plasma membrane. From these studies, we speculate that Blm3p may be a plasma membrane protein involved in the maintenance of structural integrity, cellular growth, viability and membrane permeability.

MATERIALS AND METHODS

1. Strains and plasmids.

The yeast and bacterial strains used in this work are listed in **Table 1**.

2. Media and growth conditions.

Nonsynthetic, complete solid medium (YPAD) containing 2% glucose, 2% Bacto-peptone, (Difco Laboratory, Detroit, Michigan), 1% Bacto-yeast extract (Difco), 2% Bacto-agar (Difco), and 0.16 mg/ml adenine sulfate was used for the nonselective growth of all yeast strains. Liquid nonsynthetic complete media was prepared without 2% Bacto-agar.

Solid synthetic media (SD) contains 0.2% Bacto-yeast nitrogen base (Difco), 0.5% ammonium sulfate, 2% glucose and 2.5% Bacto-agar, and were used for the selection of strains during screening steps. Specific nutritional requirements for each strain were added during the preparation of SD medium (Kaiser *et al.*, 1994). Solid synthetic medium (SD+7) contained adenine sulfate (0.480 g/100 ml), uracil (0.2 g/ 100 ml), tryptophan (1 g/100 ml), histidine (1 g/100 ml), leucine (1 g/100 ml), isoleucine (1 g/100 ml), and valine (3 g/100 ml).

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YNB-glucose (YNB-glc) minimal medium contains 6.7 g of yeast nitrogen base (without amino acids) per liter and 2% glucose, and is supplemented with 50 mg of the appropriate amino acids, and 100 mg of uracil and adenine per liter (Turchini *et al.*, 2000).

SP1 sporulation media contains 0.25% yeast extract, 0.1% glucose, 0.98% potassium acetate, 20 g/l agar and 40 mg/ml uracil, tryptophan and leucine.

The phleomycin stock solution was prepared by dissolving phleomycin (Bristol-Myers Squibb Company, Princeton, NJ) in sterile 50 mM Tris-Cl (pH 7.5) to obtain a final concentration of 10 mg/ml. Phleomycin medium was always prepared within 24 hours prior to use. Zeocin, a formulation of phleomycin D1, was prepared according to the instructions of the manufacturer (Invitrogen, Carlsbad, CA). Deletion mutants were tested on synthetic and nonsynthetic media containing 0, 3, 5, 7, 9 and 11 μg/ml of phleomycin and 200, 500, 750, 1000, 1250, 1500, and 2000 μg/ml of zeocin. Concentrations of phleomycin were determined using the Beer-Lambert equation ($OD_{245}/1.6x10^{-2}$).

3. Yeast transformation, *E. coli* transformation and plasmid isolation.

Transformation of the yeast cells was carried out using lithium acetate by the method of Ito and co-workers (1983) and as modified by Rose (1987) and Rose and Broach (1991). *E. coli* HB101 (Life Technologies, Gaithersburg, MD) was transformed according to the instructions of the manufacturer. Plasmids were partially purified from yeast transformants by the method of Hoffman and Winston (1987). The method of Birnboim and Doly (1979) was used to isolate and purify plasmids from *E. coli* transformants.

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4. Cloning of the gene by complementation.

The *blm3-1, ura3* haploid strain, CM1469-8B, was transformed with a genomic library of the yeast strain S288C. The library (kindly donated by Dr. Marian Carlson, Dept. of Genetics and Development, Columbia University, NY) was constructed by the insertion of the *Sau*3A yeast chromosomal fragments into the *Bam*HI site of the multicopy plasmid shuttle vector, YEp24. *Ura3*$^{+}$ transformants were selected after 5 days at 30°C by growth on synthetic media lacking uracil. The *Ura3*$^{+}$ transformants were replica-plated and incubated at 30°C for 3 days on supplemented SD media lacking uracil and containing 0, 3, 5, and 7 μg/ml of phleomycin. Plasmid DNA was recovered from the *Ura3*$^{+}$ transformants with increased resistance to killing by phleomycin, and amplified in *E. coli* HB101. Individual plasmid isolates were then tested for resistance to phleomycin by transforming a second *blm3-1, ura3 S. cerevisiae* strain, CM1469-5C.

5. DNA sequencing.

A mini alkaline-lysis/PEG precipitation procedure (Perkin-Elmer, Foster City, CA.) was used for obtaining sequencing-grade DNA. DNA sequencing was performed using the automated sequencer (Perkin Elmer model #373A, Foster City, CA).

6. Computer analyses.

Computer analyses were performed using PC gene, ScanProsite, BLAST, Entrez, GenBank, EMBL (European Molecular Biology Laboratory), MIPS (Munich Information Center for Protein Sequences), SGD (*Saccharomyces* Genome Database), TopPred 2 and YPD databases.

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7. *SMC1* screening.

The plasmid containing the *SMC1* gene (Stunnikov *et al.*, 1993), pAS128, was kindly provided by Dr. Douglas Koshland (Department of Embryology, Carnegie Institute of Washington, Baltimore, MD). It was constructed from the *LEU2*-based plasmid, pRS415, and was used to transform the *leu2*, *blm3-1* mutant strain, CM1469-6A.

8. Construction of the gene deletion.

Deletion of the chromosomal *BLM3* gene was carried out by the PCR deletion and replacement method described by Baudin *et al.* (1993). The basic strategy consisted of three steps: (1) PCR amplification of a gene replacement cassette that includes *HIS3* as the selectable marker; (2) transformation of yeast with the PCR product and selection of His3$^+$ recombinants; and (3) verification by PCR of the correct gene deletion using oligonucleotides complementary to the flanking sequences of the *BLM3* ORF **(Figure 4)**. The plasmid pRS303 (provided from the laboratory of Dr. Susan Henry, Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA) carries the *HIS3* replacement cassette.

Step 1 In step 1, the *HIS3* replacement cassette was synthesized by PCR, using two chimeric primers and the Bluescript-based plasmid, pRS303, as the template **(Figure 4)**. Primers were designed by National Biosciences Inc. (Plymouth, MN). Each primer contained two regions, one region which allows homologous recombination at the target locus and referred to as the deleting sequence, and a second region permitting the PCR amplification of the *HIS3* selectable marker.

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The 5' deleting sequence referred to as the oligopro (shown in upper case in **Table 2**) and the 3' deleting sequence referred to as the oligoterm (shown in upper case in **Table 2**) are flanking sequences of the *BLM3* gene. They are followed by 17 nucleotides homologous to the *HIS3* selectable marker for oligopro (shown in lower case in **Table 2**) and for oligoterm (shown in lower case in **Table 2**). PCR (PowerBlock System, Model Easy Cycler Series, Ericomp Inc., San Diego, CA) amplification was performed in a total volume of 100 μl containing 0.25 μg of pRS303, 200 μM of each dNTP, 10 μl of 10x buffer (500 mM KCl; 100 mM Tris-HCl pH 8.8; 15 mM $MgCl_2$; 1% Triton X 100; 0.1% gelatin), 0.5 μM of each primer and 2.5 units of Taq polymerase (Perkin Elmer, Foster City, CA). The reaction mix was incubated for 5 minutes at 95°C and submitted to 2 cycles of PCR (30 sec at 94°C, 30 sec at 45°C and 2 min at 74°C) followed by 30 cycles (30 sec at 94°C, 30 sec at 50°C and 2 min at 74°C). In the final cycle, the extension step was for 5 min.

Step 2 In step 2, one μg of the PCR product, 1.1 kb long, was directly transformed into the *BMA1* diploid *his3Δ200/his3Δ200* strain (kindly donated by Dr. Agnes Baudin, Dept. of Molecular Genetics, Centre de Genetique Moleculaire, Cedex, France). This strain lacks all sequences corresponding to the *HIS3* gene included in the PCR product, and thereby prevents gene conversion and homologous recombination at the *HIS3* locus.

Step 3 In step 3, the transformants were tested by PCR for the replacement of the *BLM3* gene by the *HIS3* gene **(Figure 4)**. Test primers 1 and 2 (**Table 2**) were designed by National Biosciences Inc. (Plymouth, MN) from the flanking

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sequences of the *BLM3* ORF. PCR amplification was performed in a total volume of 100 μl containing 0.15 μg of genomic DNA isolated using the High Pure PCR Template Preparation Kit (Boehringer Mannheim Inc., Indianapolis, IN) according to the instructions of the manufacturer. The reaction mix was incubated for 10 minutes at 94°C and submitted to 3 cycles of PCR (10 sec at 94°C, 30 sec at 55°C and 10 min at 68°C), followed by 22 cycles (10 sec at 94°C, 30 sec at 55°C and 8 min at 68°C), then followed by 3 cycles (10 min at 72°C). The heterozygous diploids bearing the null allele of the *BLM3* gene (*BLM3/blm3Δ::HIS3*) were sporulated on SP1 sporulation media at 22°C for 7 days.

9. Construction of Blm3p-GFP fusion.

A modification of the existing protocol (Baudin *et al.*, 1993) used to replace and delete *BLM3* (as described in Materials and Methods section 8) was used to fuse GFP to the 3' end of *BLM3*. The basic strategy consists of three steps: (1) PCR amplification of the cassette containing the GFP coding sequence and the selectable marker (*HIS3*); (2) transformation of yeast with the PCR product and selection for His3+ transformants; and (3) verification by PCR of correct GFP integration using a universal test primer in GFP or HIS3 and a test primer in the target gene sequence (3'end of *BLM3*).

Step 1 In step 1, a cassette containing the optimized S65T mutant version of the green fluorescent protein (GFP) coding sequence and the selectable marker (*HIS3*) was synthesized by PCR (Niedenthal *et al.*, 1996), using two chimeric primers and the Bluescript-based plasmid, pBM3412 (which will not replicate in

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yeast, thereby reducing background from yeast transformants containing template plasmid). The cassette was kindly donated by the laboratory of Dr. Mark Johnston (Washington University, St Louis, MO). The cassette was amplified by PCR using one forward primer (shown in upper case in **Table 3**) containing the last 45 bases of the *BLM3* coding sequence (excluding the stop codon) and the first 18 bases of the GFP coding sequence (shown in lower case in **Table 3**), and one reverse primer containing 45 bases of homology downstream from the 3' end of *BLM3* about 20 nucleotides away from the 3' end of the *BLM3* coding sequence (shown in upper case in **Table 3**) and 18 bases in the *HIS3* untranslated region (shown in lower case in **Table 3**).

Step 2 In step 2, one μg of the PCR product, 1.9 kb long, was transformed into yeast strain, CM1522-9A (*his3Δ200*), a segregant of CM-1522 (**Table 1**) carrying a complete deletion of all the sequences homologous to the *HIS3* gene. His+ transformants were isolated on supplemented SD media lacking histidine. All His+ transformants were checked for phleomycin resistance (to make sure the Blm3p has not lost its activity) on SD media containing 0, 3, 5, 7 and 9 μg/ml of phleomycin.

Step 3, In step 3, correct GFP integration was verified by PCR of His+ transformants, using a forward test primer (20 bases long) about 680 bases upstream from the end of the *BLM3* gene (**Table 3**) and a reverse test primer (20 bases long) in the *HIS3* gene (**Table 3**). Only correctly integrated transformants yield a PCR product. PCR amplification was performed in a total volume of 100 μl containing 0.15 μg of genomic DNA isolated using the High Pure PCR

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Template Preparation Kit (Boehringer Mannheim Inc., Indianapolis, IN) according to the instructions of the manufacturer. The reaction mix was incubated for 10 minutes at 94°C and submitted to 3 cycles of PCR (10 sec at 94°C, 30 sec at 55°C and 10 min at 68°C) followed by 22 cycles (10 sec at 94°C, 30 sec at 55°C and 8 min at 68°C), then followed by 3 cycles (10 min at 72°C).

To induce Blm3p expression in Blm3p-GFP tranformants, phleomycin (5 μg/ml) and methyl methansulfonate (MMS, 0.1%) was added for 1 hour to cells grown in liquid YPAD overnight at 30° C.

10. Microscopy.

GFP fluorescence was observed in Blm3p-GFP cells. Cells were viewed using a Zeiss Axioskop microscope at 590 nm. Blm3p-GFP cells were also fixed on polylysine-coated cover slips and examined using confocal laser scanning microscopy (Molecular Dynamic Sarastro 2000 CLSM) at 590 nm .

BLM3, blm3-1 and *blm3Δ::HIS3* cell sizes were observed using light microscopy (Nikon HFX-II).

11. Mitotic growth and cell size assay.

The *BLM3, blm3-1 and blm3Δ::HIS3* strains were grown in liquid YPAD at 30°C overnight, plated on solid medium for viable cell counts and checked for cell size using light microscopy (Nikon HFX-II). Strains were also grown in liquid YPAD for 72 hours. Cell counts were taken every 90 minutes using a hemacytometer.

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12. Assay for respiratory deficiency.

Mutant *blm3-1* colonies grown on solid YPAD at 30°C were overlayed with 100 ml of 2,3,5-triphenyltetrazolium chloride (TTC) agar (1.5 % Bacto agar in 0.067 M phosphate buffer at pH 7.0; 0.1% with respect to TCC) and incubated for 2 hours at 30°C (Ogur *et al.*, 1957).

13. Viability Assay.

BLM3, *blm3-1* and *blm3Δ::HIS3* cells were grown exponentially in liquid YPAD from 10^4 cells/ml to 6 x 10^7 cells/ml (cell counts were taken by hemacytometer), suspended in deionized water, plated on YPAD (100 cells/plate on 5 separate plates), grown for two days at 30°C and colonies were counted.

14. Cation treatments.

BLM3, *blm3-1*, and *blm3Δ::HIS3* strains were grown exponentially from 1 x 10^4 cells/ml to 4 to 6 x 10^7 cells/ml (cell counts were taken by hemacytometer) in liquid YPAD. The cells were centrifuged and the supernatants were removed to yield cell pellets of 100 µl. To each cell pellet, 50 µl of one of the following solutions was added: 2 M NaCl, 2 M KCl, 2 M $NiCl_2$, 2 M $MgCl_2$, 2 M $CrCl_3$ or 1.5 M $FeCl_3$. The cell suspensions were incubated for 10 min at 30°C without shaking and centrifuged. The supernatants were removed using a Pasteur pipette. The cell pellets were diluted in 5 ml of a 0.1 M NaCl saline solution and plated on solid YPAD media for viable cell counts. Samples of cells treated with the saline solution were used as a control. All viable cell counts were calculated relative to this control.

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15. SDS treatments.

BLM3, *blm3-1, and blm3Δ ::HIS3* strains were grown in liquid and on solid YNB-glucose (2%) and YNB-glucose (2%) supplemented with 0.005% sodium dodecyl sulfate (SDS) at 30°C.

16. Calcofluor treatments.

BLM3, *blm3-1* and *blm3Δ::HIS3* strains were grown on complete SD medium supplemented with 0, 20, and 80 μg/ml of calcofluor at 30°C for 3 days.

17. Northern blot analysis.

For northern blot analysis, the 5.4 kb *BLM3* DNA fragment was isolated by PCR as described above and separated by electrophoresis on a 1% agarose gel. The 5.4 kb fragment was isolated from the gel using the QIA quick Gel Extraction Kit (Qiagen Inc., Santa Clarita, CA) according to the instructions of the manufacturer. The *BLM3* isolate was labeled with ^{32}P using the Random Primed ^{32}P DNA Labeling kit (Boehringer Mannheim, Indianapolis, IN) and hybridized to the Human RNA Master Blot (Clontech, Palo Alto, CA) according to the instructions of the manufacturer.

RESULTS

Part 1. Cloning by complementation.

1. Cloning of the *BLM3* gene.

The *S. cerevisiae* yeast genomic library (S288C) in the multicopy plasmid shuttle vector, YEp24 (**Figure 1-1**), was screened for plasmids complementing the hypersensitivity to killing by phleomycin conferred by the *blm3-1* mutation. Fifty-seven hundred yeast transformants bearing the *URA3* marker, and thus able to grow

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on synthetic media lacking uracil, were replica-plated onto synthetic media containing 0, 3, 5, 7 and 9 μg/ml phleomycin to determine resistance to killing by the drug. A recombinant plasmid (pPM311) was isolated from one of the *URA3* transformants (M13-95) that was resistant to killing by phleomycin up to 7 μg/ml (**Figure 1-2 lane 3 row 4 versus rows 2 and 3**) and amplified in *E. coli*. All *E. coli* transformants were tetracycline sensitive since the yeast chromosomal or library DNA was inserted at the BamHI site within the tetracycline gene of the YEp24 plasmid (**Figure 1-1**) in the *S. cerevisiae* library. These results were reconfirmed on LB plates containing tetracycline. After amplification in *E. coli*, the pPM311 plasmid retained its capacity to fully complement the hypersensitivity to killing by phleomycin conferred by the *blm3-1* mutation. This was demonstrated when pPM311 was transformed into a second *blm3-1, ura3-1* strain (**Figure 1-2**; **lane 3, row 5 versus rows 2 and 3**). This confirmed it was the plasmid complementing the hypersensitivity to killing by phleomycin conferred by the *blm3-1* mutation and not a reversion..

Part 2. Computer analyses.

1. Computer analyses of *BLM3*.

The DNA sequence similarity searches of all databases using the BLAST program showed that the DNA sequence of the insert in the recombinant plasmid was localized to chromosome VI. The DNA insert was found to encompass an open reading frame encoding approximately 57% of the amino terminus of an unknown gene (SGD accession # YFL007W) and the carboxy half of the *SMC1* gene (Strunnikov *et al.*, 1993) of yeast (**Figure 2-1**). We speculated that the region

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complementing the *blm3-1* mutation was probably from the amino terminus region of the unknown gene rather than the carboxy half of the *SMC1* gene since the *SMC1* portion did not contain the amino portion of the gene containing the promoter region. The unknown gene was identified as the *BLM3* gene based on genetic analyses presented below in Part 3 section 2. Computer analysis (MIPS database) based on functional assignment predicts Blm3p to function in cell growth, cell division and DNA synthesis.

Analyses using the ScanProsite database were carried out to determine if additional motifs could be identified in the Blm3p. The hydrophilic regions, which are predicted to exist outside the membrane, contain a total of ninety-five putative motifs. They include three leucine zippers, three cAMP/cGMP-protein kinase phosphorylation sites, five tyrosine kinase phosphorylation sites, thirteen n-myristoylation sites, sixteen n-glycosylation sites, twenty-one protein kinase C phosphorylation sites and thirty-four casein kinase II phosphorylation sites.

Computer analyses according to Nelissen *et al.* (1997) suggest that Blm3p may be a new member of the major facilitator superfamily (MFS) of permeases involved in transport. Interestingly, leucine zippers have been identified in several proteins containing multitransmembrane spanning domains involved in transport (Abejon *et al.*, 1995; Eckhardt *et al.*, 1996).

2. *SMC1* Screening.

Since the carboxy half of the *SMC1* gene resides on the pPM311 recombinant plasmid that functionally complemented the *blm3-1* mutation, we tested the ability of the entire *SMC1* gene to complement the phleomycin hypersensitivity conferred by

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the *blm3-1* mutation. A *blm3-1*, *leu2* strain was transformed with the pAS128 plasmid bearing the entire *SMC1* gene (**Figure 2-1**). One hundred forty-seven *Leu*$^+$ transformants were isolated. Seventy-five of these transformants were screened for resistance to killing on synthetic media containing 0, 3, 5, 7 and 9 μg/ml of phleomycin. All seventy-five exhibited hypersensitivities comparable to *blm3-1* mutant cells (e.g. **Figure 2-2; lane 3, rows 2, 3 and 4**), indicating that *SMC1* did not functionally complement the *blm3-1* mutation in these cells.

3. Predicted amino acid sequence of Blm3p.

The DNA insert encoding *BLM3* contains an open reading frame predicting a protein of 1804 amino acid residues (**Figure 2-3, A**) with a molecular mass of 207.6 kDa. Homology searches using the BLAST and PSI-BLAST programs of all databases showed no significant homology with any known protein in yeast or any other organism, indicating that the protein encoded by *BLM3* is novel.

Hydropathy analysis by the Klein, Kanehisa and Delisi (Klein *et al.*, 1985) and Kyte and Doolittle (Kyte and Doolittle, 1982) methods suggested the Blm3p is an integral multitransmembrane protein (**Figure. 2-3, B**.). PC gene and Entrez databases predicted a protein structure with seven to ten transmembrane domains, respectively. These domains are illustrated in **Figure. 2-3, A**. These findings prompted the investigation of Blm3p as a membrane protein.

4. Comparison of Blm3p to other proteins characterized as having transport functions.

In addition, three classified proteins, Rud3p (YOR216c, P-value 0.63), Pex3p (YDR329c, P-value 0.98) and Vps33p (YLR396c, P-value 0.98) were

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identified using SGD BLASTP. They exhibited weak homology to Blm3p and are involved in vesicle, peroxisomal and vacuole transport respectively. P values range from "worst" to best" (1.0 to 1e-10 to 1e-200 to 0.0).

Part 3. Partial characterization of *BLM3*.

1. Localization of Blm3p.

A global subcellular localization study was conducted on all the yeast ORFs (Snyder and co-workers at the Yale Genome Analysis Center, New Haven, CT) carrying an influenza virus hemagglutinin epitope transposon-tag (HAT tag). The tagged Blm3 protein examined by indirect immunofluorescence using monoclonal antibodies directed against the transposon-encoded HA epitope localized Blm3p inside the nucleus. These results seemed very unlikely based on computer analyses, which predicted Blm3p to be a hypothetical membrane protein. Thus, the rationale for proceeding with a localization study in this project was to confirm where Blm3p localized. These localization studies were conducted using GFP.

Blm3p-GFP fusions were constructed as described in Materials and Methods. The advantage of this method is that the GFP fusion created is stably integrated at the normal chromosomal locus so that it can be expressed at normal levels. A GFP-HIS3 cassette cloned into pBM3412 was amplified by PCR. Primers were designed to target GFP to the 3'end of *BLM3* (**Table 3**), which appeared to be the region not necessary for complementation of the *blm3-1* mutation (**Figure 2-1**). The 1.9 kb PCR product (**Figure 3-1**) was transformed into the CM1522-9A (*his3Δ200*) haploid strain. Two His+ phleomycin resistant Blm3p-GFP fusion transformants (CM1522-9A containing *blm3::gfp-HIS3*) (**Figure 3-2**) that contained the correctly integrated

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PCR products (2.0 kb) were isolated (**Figure 3-3**). Using confocal and fluorescence microscopy, weak levels of expression of the Blm3p-GFP appeared to be localized around the cell surface in approximately 25% of the cells (data not shown).

The weak expression levels observed for the Blm3p fusions may be due to low expression levels of Blm3p in the cell. In fact, Dr. Leonia Samson (Harvard University, Boston MA, personal communication) and the TRIPLES database, (Yale Genome Analysis Center, New Haven, CT) reported that Blm3p is expressed at low levels in *S. cerevisiae* (∠1 molecule/cell). To try to induce higher Blm3p expression, phleomycin (5 μg/ml) and (MMS, 0.1%), an alkylating agent reported to induce Blm3p expression (Jelinsky and Samson, 1999), was added to Blm3p-GFP cells for one hour at 30°C. Phleomycin (**Figure 3-4**) and MMS (data not shown) however, did not have much of an effect on the Blm3p-GFP level of expression compared to cells without phleomycin and MMS (data not shown).

The most recent localization studies (conducted by Snyder and co-workers at the Yale Genome Analysis Center) examined by indirect immunofluorescence using monoclonal antibodies directed against the transposon-encoded HA epitope. The group has now localized Blm3p to the outer membrane with weak staining (comparable to background levels) in about 25 % of the cells. Weak staining is probably due to weak expression levels of Blm3p. Thus, these recent findings corroborate the GFP localization studies.

2. Construction of the *BLM3* null mutation.

Functions of genes and proteins can be determined by analyses of mutations, particularly by deletion mutations (Lorenz *et al.*, 1995). For this purpose, gene

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deletions can be created by homologous recombination (Ivic *et al.*, 2000). A *BLM3* null mutation was created with the rationale of it assisting in the functional characterization of the gene. The null allele of *BLM3* was constructed as described in detail in Materials and Methods. The *HIS3* disruption cassette in the pRS303 *HIS3*-based plasmid (designed to delete *BLM3*) was amplified by PCR. The 1.1 kb PCR product (**Figure 3-5 lanes 4-8**) was transformed into the *BMA1* diploid strain (*his3Δ200/his3Δ200*). Five diploid strains, CM-1521, CM-1522, CM-1523, CM-1524, and CM-1525, bearing the null allele of *BLM3* in the heterozygous configuration *(BLM3/blm3Δ::HIS3)* were created (**Figure 3-6 lanes 4-8**) and compared to homozygous *BLM3*/*BLM3* and *blm3-1*/*blm3-1* strains for their overall phleomycin resistance. Interestingly, all heterozygous diploids exhibited intermediate resistance (data not shown).

3. Viability and drug resistance of *BLM3* null mutants.

Ten tetrads from one of the sporulated HIS^+ prototrophic diploids (CM-1522) were dissected. All the segregants were viable (e.g. **Figure 3-7, A**) and exhibited 2:2 segregation for the *HIS3* marker (**Table 3-1**), proving that the gene deleted and replaced by *HIS3* is not an essential gene. The 2:2 segregation of the His^+ and histidine-requiring segregants from two complete tetrads is illustrated in **Figure 3-7, B**.

Growth of His^+ (*blm3Δ::HIS3*) and histidine-requiring (*BLM3*) segregants were tested in three independent experiments on synthetic and nonsynthetic media containing phleomycin (7 μg/ml). None of the histidine-requiring segregants displayed hypersensitivity to killing by phleomycin (**Table 3-1**). However, all of the

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His$^+$ segregants displayed hypersensitivity to killing by phleomycin (**Table 3-1**). These results indicated that the targeted *BLM3* gene was disrupted by the deletion, and that the *BLM3* gene is not essential for viability in the absence of the bleomycin-phleomycin group of antibiotics. The *blm3Δ::HIS3* mutant cells however, exhibit less hypersensitivity in the presence of phleomycin than *blm3-1* mutant cells compared to *BLM3* strains of related genetic backgrounds.

4. Cell Wall Assay.

Although computer analyses revealed that Blm3p is a potential membrane protein, localization studies suggested that Blm3p appears around the cell surface. Thus, assays for cell wall function and cell membrane integrity were carried out. Cell wall mutants are sensitive to calcofluor (Roncero *et al.*, 1988), a compound that interacts with chitin, interferes with cell wall assembly and growth, and is toxic to yeast cells (Roncero *et al.*, 1988). Bleomycin also damages yeast cell walls (Moore *et al.*, 1992; Beaudouin *et al.*, 1993), and may have selected for a cell wall mutant during the isolation of the original *blm3-1* mutant (Moore, 1980 and 1991). *BLM3* and *blm3-1* strains were grown on complete SD medium supplemented with calcofluor. The results are presented in **Table 3-2**. The *BLM3* and *blm3-1* strains all grew equally well in the presence and absence of calcofluor, suggesting that the mutant strains do not have an alteration in their cell wall that could be detected by this assay. Thus, the Blm3p is probably not a cell wall protein. A *blm3Δ::HIS3* mutant strain was also tested and compared to a related *BLM3* strain. Both strains also grew equally well in the presence and absence of calcofluor (**Table 3-2**). This phenotype after growth in the presence of calcofluor was also demonstrated for the *BLM3*

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disrupt created by Snyder and co-workers (Yale Genome Analysis Center, New Haven, CT)

5. Northern blot analyses.

Using the entire 5.4 kb *BLM3* gene as a probe, a homologue was sought in a variety of human tissues by northern blot analysis. A human RNA master tissue blot containing mRNA from fifty different human adult and fetal tissues was examined. Cross hybridization was anticipated since the entire *BLM3* gene was used. In adult tissues, high and low levels of hybridization were obtained (**Table 3-3**). However, no hybridization was detected in any of the fetal tissues. The significance of these results is not immediately apparent because the specific regions of hybridization to *BLM3* were not determined, but the results may suggest that the region or regions of *BLM3* that hybridized in the adult tissues may be of fundamental importance in development after 36 weeks since they were not expressed in the 16 to 36-week fetal tissues.

6. Additional phenotypic analyses.

Several additional phenotypes were analyzed and compared among *BLM3*, *blm3-1* and *blm3Δ::HIS3* strains.

a. Colony and cell size differences of *blm3-1* cells compared to *BLM3* cells.

Smaller colonies were observed for several *blm3-1* mutant strains than for a related *BLM3* strain after the strains were grown on YPAD plates (data not shown). In addition, several *BLM3* strains and *blm3-1* strains were grown in liquid YPAD and observed by light microscopy. Mutant cells were always smaller than the related *BLM3* cells. A comparison of mutant cells and *BLM3* cells is illustrated in **Figure 3-**

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8, A and B. However, there were no significant differences in colony sizes (data not shown) or cell sizes between *blm3Δ::HIS3* null mutants and *BLM3* cells. A comparison of *blm3Δ::HIS3* cells compared to related *BLM3* cells is illustrated in **Figure 3-8, C and D**.

b. Assay for respiration deficiency.

A variety of mutations have been identified that disturb membrane integrity. They include mutations in genes involved in respiration deficiency (Taber *et al.*, 1987) as well as genes involved in the maintenance of structural integrity of the membrane (Taber *et al.*, 1987; Ichige and Walker, 1997). During respiration deficiency, caused by functionally defective mitochondria, small colonies with defective growth rates form (Fergurson and Turner, 1988; Fergurson and Borstel, 1992). To determine if the small colony size observed for *blm3-1* cells was due to respiration deficiency, *blm3-1* colonies grown on solid YPAD were overlayed with TTC. White colonies observed after TTC overlay represent respiration deficiency, while red colonies are respiration proficient (Raut, 1953). All the *blm3-1* colonies observed after TTC overlay were red, and thus were respiratory proficient. These results suggest that the smaller colony size observed for the *blm3-1* mutant is not due to respiratory deficiency. It also seems likely that any disturbance in membrane integrity displayed in the *blm3-1* mutant compared to *BLM3* is not due to respiratory deficiency.

c. Mitotic growth.

Alterations in the plasma membrane have been associated with changes in cellular growth rate and viability in yeast cells (Mager and Varela, 1993; Skaggs *et*

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al., 1996). Several *blm3-1* mutant segregants exhibited reduced viability and rates of growth in comparison to *BLM3* wild-type strains during cloning and partial characterization of the *BLM3* gene. These observations led to further studies of viability and growth of *BLM3*, *blm3-1* and *blm3Δ::HIS3* strains. In the viability analyses, *blm3-1* mutant cells displayed decreases in viability compared to *BLM3* cells. The viability of the *blm3-1* mutant strain, (CM1469-5C) compared to wild-type *BLM3* (CM1469-5A) is illustrated in **Figure 3-9** for three separate experiments. The *blm3Δ::HIS3* (CM1522-9B) and *BLM3* (CM1522-9A) strains were also tested, but no significant changes in viability were displayed for both strains (data not shown).

To compare growth rates, *BLM3* (CM1469-5A) and mutant *blm3-1* (CM1469-5C) cells were grown in liquid YPAD at 30°C, and cell densities were recorded about every 90 minutes for 72 hours. The *blm3-1* cells were found to grow slower than the *BLM3* cells during logarithmic growth (**Figure 3-10, A**), indicating a reduced growth rate of mutant cells compared to the *BLM3* cells. The growth rates of mutant *blm3Δ::HIS3* (CM1522-9B) and the related wild-type *BLM3* (CM1522-9A) cells were also compared, but no significant differences were observed (**Figure 3-10, B**).

7. Membrane Integrity Studies.

It has been shown that exposure of yeast cells to high osmolarities leads to alterations in the plasma membrane involving the collapse of ion gradients, dehydration, and decrease in cellular viability (Mager and Varela, 1993). Based on the working hypothesis that Blm3p could be a plasma membrane protein, further studies were undertaken to investigate plasma membrane integrity by using high

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osmotic solutions such as 2 M cations (Bard *et al.*, 1978) and the strong membrane destabilizing agent for the plasma membrane, SDS (Turchini *et al.*, 2000).

a. Cation induced death

The rationale for the next experiments was to determine if membrane permeability among the *BLM3* wild-type cells differed from that in *blm3-1* and *blm3Δ::HIS3* mutant strains. Experiments were carried out by incubating cells for 10 min with 2 M NaCl, 2 M KCl, 2 M $MgCl_2$, 2 M $CaCl_2$, 2 M $CrCl_3$ and 1.5 M $FeCl_3$. *BLM3* and *blm3-1* strains were tested in three separate experiments. Viability was calculated for *BLM3* and *blm3-1* strains after exposure to each cationic solution. Results for each experiment are illustrated in **Figure 3-11**. Pulses of $FeCl_3$ and $CrCl_3$ resulted in ≥99% killing for all strains, and thus the results for these cations are not included in **Figure 3-11**. Mutant *blm3-1* strains displayed significantly higher viability than *BLM3* cells in the presence of Na^+ and K^+, but not to Mg^{2+} or Ca^{2+}, in every experiment. From these results we concluded that *blm3-1* cells may be more resistant than *BLM3* cells to Na^+ and K^+. On the other hand, the viability of the *blm3Δ::HIS3* strains (CM1522-9B and CM1522-9C) in the presence of Na^+, K^+, Mg^{2+} Ca^{2+} was not significantly different from that of related *BLM3* (CM1522-9A and CM1522-9D) strains in two separate experiments **(Figure 3-12).** Pulses of $FeCl_3$ and $CrCl_3$ resulted in ≥99% killing for all four strains, and thus the results for these cations are not included in **Figure 3-12**.

b. SDS membrane alteration assay.

To further characterize *blm3-1* and *blm3-1Δ::HIS3* mutant strains, their viability was compared with *BLM3* strains with and without 0.005% SDS. *BLM3* and

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mutant *blm3-1* cells grew without SDS on day two **(Figure 3-13)**. However, *blm3-1* cells exhibited higher resistance than *BLM3* cells after growth with SDS. Mutant *blm3-1* colonies grew on medium containing SDS after two days, although they were smaller than those grown without SDS **(Figure 3-13)**. In contrast, *BLM3* colonies appeared after two days growth in the absence of SDS (**Figure 3-13**), but not in the presence of SDS (data not shown). After five days, however, microcolonies were observed on SDS containing media (**Figure 3-13**). The same results were obtained in liquid assays for growth in the presence and absence of SDS, as summarized in **Table 3-4**. From these preliminary observations, we concluded that *blm3-1* cells may be more resistant than *BLM3* cells to the destabilizing effects of SDS. When the same tests were carried out on a *blm3Δ::HIS3* strain and a related *BLM3* segregant, the strains appeared to grow equally well in the presence and absence of 0.005% SDS (data not shown).

DISCUSSION

1. Blm3p is a novel membrane protein..

The *BLM3* gene was isolated from a *S. cerevisiae* genomic library by complementing the hypersensitivities to killing by phleomycin and bleomycin that are conferred by the codominant *blm3-1* mutation. Computer-aided analysis suggests that Blm3p is a novel integral membrane protein. Using the GFP tagging approach, Blm3p appeared to be localized around the outer cell surface and weakly expressed. The localization studies using a transposon tagging approach conducted by Snyder and co-workers (TRIPLES database) initially localized Blm3p to the nucleus, but recently localized Blm3p to the outer cell membrane with weak staining over

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background levels. The latter result supports the GFP findings in the current project. The weak expression levels observed for Blm3p in this project and the recent Yale study corroborate the findings of Leonia Samson and co-workers (Harvard University School of Public Health, Boston, MA) and Snyder and co-workers (TRIPLES database) that Blm3p is expressed at low levels in *S. cerevisiae* (∠1 molecule/cell). It is not known if *BLM3* is an inducible gene that may be expressed at high levels in the cell under specific conditions when it is needed. Yet even with low expression we found that *blm3-1* mutant cells are smaller in size, grow slowly, exhibit reduced viability, are hypersensitive to killing by phleomycin and bleomycin, and exhibit increased viability in the presence of $Na^{+}$and K^{+} (2 M) and SDS (0.005%) compared to *BLM3* cells. Interestingly, computer analyses (MIPS database) based on functional assignment predicts Blm3p to function in cell growth, cell division and DNA synthesis.

2. The *blm3-1* and *blm3-1Δ ::HIS3* mutant strains are hypersensitive to killing by bleomycin and phleomycin.

Haploid and diploid yeast cells containing the original *blm3-1* mutation and haploid *blm3-1Δ ::HIS3* cells are more sensitive to killing by bleomycin and phleomycin than cells containing the *BLM3* gene. Membrane alteration has been associated with drug resistance in bacteria (Abadi *et al.*, 1996). A possible mechanism for *BLM3* resistance to bleomycin and phleomycin in *S. cerevisiae* may involve a reduction in the drug's ability to enter the cell because of a change in the structure of the outer membrane.

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Hypersensitivity in the presence of phleomycin demonstrated in *blm3-1* and *blm3Δ ::HIS3* cells may also be due to changes in the membrane that lead to an altered checkpoint and the inability of the cells to undergo proper DNA repair and growth. Changes in the regulation of the cell cycle, however, remain to be determined.

X-ray induced apoptosis in human cells have shown alterations in membrane integrity affecting membrane permeability and membrane potential (Ferlini *et al.*, 1999). Changes in the membrane potential have also been associated with the uptake of radiomimetic bleomycin into cells. Electrochemotherapy is a new antitumor clinical treatment that delivers bleomycin with electric pulses to tumor sites (Domenge *et al.*, 1996). This treatment permeabilizes the cells in the tumor, allowing bleomycin to enter the cells and kill them. Electropermeabilization exposes cells to short intense electric pulses. These transient and reversible electric impulses cause a change in the normal membrane potential (Zimmermann, 1982). This change allows the membrane to undergo restructuring which makes the membrane permeable to molecules that would otherwise be blocked from entering cells. This change in the membrane is characterized by the formation of porous structures, which allow for the exchange of materials (Zimmermann, 1982). Membrane potential may also be altered in *blm3-1* cells, allowing more bleomycin or phleomycin to enter the cells than to enter *BLM3* cells. An altered Blm3p may be mimicking the effects on the membrane after electropermeabilization, and allow for higher drug uptake than in *BLM3* cells. Understanding more about electrochemotherapy and the delivery of bleomycin into

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human tumor cells after exposure to electric pulses may lead to new knowledge on the bleomycin internalization process. In yeast, this process may involve Blm3p.

3. Membrane integrity and phenotypes of *blm3-1* cells.

The cell membrane has been postulated to act as a "pleiotypic mediator" involved in coordinating responses to metabolically unrelated biochemical reactions inside the cell (Herschko *et al.*, 1971). One of these biochemical reactions involves cell cycle control. The cell cycle involves cell growth and checkpoint mechanisms for DNA repair. Herschko and coworkers (1971) suggested that loss of cell cycle regulation results from changes in the membrane. Several yeast genes encoding membrane proteins involved in cell membrane integrity have been speculated to coordinate the decisions regarding cell cycle progression that result from changes in cell membrane integrity (Kuo and Grayhack, 1994). Compared to *BLM3* cells, mutant *blm3-1* cells grow more slowly and are less viable, yet they display increased viability in the presence of $Na^+$and K^+ cations (**Figure 3-11**) and SDS (**Table 3-4, Figure 3-13**). Normally yeast cells exposed to high osmolarities display a decrease in cell viability and alterations in their plasma membrane (Mager and Varela, 1993). It is possible that the increased resistance to $Na^+$and K^+ cations (**Figure 3-11**) and SDS (**Table 3-4, Figure 3-13**) displayed by *blm3-1* strains may involve a structural change at the level of the membrane surface allowing the mutant cells to grow better than *BLM3* cells in the presence of $Na^+$and K^+ cations (**Figure 3-11**) and SDS (**Table 3-4, Figure 3-13**). Blm3p may be involved in the maintenance of structural integrity of the plasma membrane, which has an affect on viability.

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4. The *blm3-1* mutant cells are smaller than *BLM3*.

Mutant *blm3-1* cells are smaller in size compared to *BLM3* cells. However, there is no significant change in size for *blm3-1Δ ::HIS3* cells compared to *BLM3* cells. Johnston and co-workers (1979) proposed that a cell, in spite of growth conditions, has to attain a critical cell size before cell division. Regulation of this critical size occurs within G1. Yeast *wee* mutants that have small cell sizes and an altered cell-cycle behavior have been shown to have a longer G1 period and a shortened S + G2 + M period (Nasmyth, 1979). These mutants have lost their size control over nuclear division and are accelerated into mitosis shortly after the completion of S phase, thus dividing at a smaller size (Nurse and Thuriaux, 1977). Cells that have been accelerated into division take a longer time than usual to complete the next cell cycle (Smith and Mitchison, 1976), which seems likely to have an effect on the entire cell cycle pathway involving checkpoint, DNA repair, cell viability and growth rate. The mutant *blm3-1* cells may have an alteration in their cell cycle affecting cellular viability and growth rate, although this has not been investigated.

5. Smaller colony sizes of *blm3-1* cells are not due to respiration deficiency.

Smaller colony sizes were observed for *blm3-1* cells compared to *BLM3* cells grown on YPAD plates (data not shown). During respiration deficiency, small colonies with defective growth rates form due to functionally defective mitochondria (Ferguson and Turner, 1988; Ferguson and Borstel, 1992). According to TTC overlay studies, the *blm3-1* smaller colony sizes do not appear to be due to respiration deficiency. Since disturbances in membrane integrity have been shown to be

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associated with mutations in genes involved in respiration deficiency (Taber *et al.*, 1987) as well as genes involved in the maintenance of structural integrity (Taber *et al.*, 1987; Ichige and Walker, 1997), and the *blm3-1* cells appear to be respiratory proficient, the smaller colony sizes may be due to changes involving the maintenance of structural integrity.

6. Different phenotypes exhibited in the *blm3-1* and *blm3-1Δ ::HIS3* strains.

Compared to *BLM3* cells, mutant *blm3-1* cells are more sensitive to phleomycin, more resistant to $Na^+$and K^+ cations (**Figure 3-11**) and SDS (**Table 3-4, Figure 3-13**), smaller in cell size, and grow at a slower rate. Mutant *blm3Δ ::HIS3* cells compared to *BLM3* cells, on the other hand, are not more resistant to $Na^+$and K^+ cations (**Figure 3-12**), and SDS (0.005%) (data not shown), are not smaller in size and do not grow slower. Thus, phenotypes under these conditions for mutant *blm3Δ ::HIS3* cells are similar to *BLM3* cells. These changes in phenotypes exhibited by the *blm3-1* and *blm3Δ ::HIS3* cells with respect to cation induced death, SDS sensitivity, cell size and growth rate, may be due to *BLM3* cells being non-essential for growth. The *blm3Δ ::HIS3* cells may have means of compensating for changes in cell size, viability, and growth, whereas cells containing the *blm3-1* mutation synthesize abnormal Blm3p which alters cell size, viability, and growth. In yeast, it has been shown that null phenotypes can be compensated for by the expression of other proteins. For example, P-glycoprotein in *S. cerevisiae*, can suppress the null *ste6* mutation (Ruetz *et al.*, 1994). Also, increased expression of the translation elongation factor 1α in *S. cerevisae* bypasses the lethality conferred by a null allele of *TEF5, which* encodes another elongation factor 1β (Kinzy and Wollford, 1995). Thus, it

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may be possible that another protein and/or pathway within the cell is able to compensate for the *blm3Δ* phenotypes, or increased expression of another protein may bypass the effects of the *blm3Δ* phenotypes, allowing the *BLM3* cells to function like wild-type. A transposon insertion of *BLM3* created by Synder and co-workers (Yale Genome Analysis Center, Yale University, New Haven, CT) also displays phenotypes similar to the wild-type strain for cation sensitivity, SDS sensitivity, calcofluor and growth. Their results for the disrupted *BLM3* gene corroborate the similar phenotypes exhibited for *BLM3* and *blm3Δ::HIS* cells in the current project.

7. While computer aided analyses suggest Blm3p is a permease, its role as a transporter remains to be determined.

Although the current work leads to the model that Blm3p is a novel plasma membrane protein involved in the maintenance of membrane integrity, cell growth, cell size, and viability, its role as a transporter cannot be ruled out. The possible role of Blm3p as an antiporter and/or MDR protein is discussed.

The MFS of permeases are present in bacteria, archaea, and eukarya, and contain members that can function by solute uniport, symport, and antiport with inwardly and/or outwardly directed polarity (Pao *et al.*, 1998). MFS permeases are involved in the transport of simple sugars, oligosaccharides, inositols, drugs, amino acids, nucleosides, organophosphate esters, Krebs cycle metabolites, and a large variety of organic and inorganic anions and cations (Pao *et al.*, 1998).

a. The possible role of Blm3p as an antiporter.

An antiporter, Nha1p, found in *S. cerevisiae* mediates sodium and potassium efflux with external H^+ (Banuelos *et al.*, 1998; Navarre and Goffeau, 2000). Nha1p

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like Blm3p is estimated to be ∠ 1 molecule/cell (TRIPLES database, Yale Genome Analysis Center, New Haven, CT; Dr. Leonia Samson, Harvard University, Boston MA, personal communication), and is approximately half the size of Blm3p. *NHA1* is non-essential like *BLM3*. Based on the viability and Na^+ and K^+ results for *blm3-1* compared to *BLM3* cells, Blm3p might be an antiporter involved in sodium and potassium efflux like Nha1p.

K^+ is the most abundant cation in *S. cerevisiae* cells as well as in most other eukaryotic and prokaryotic organisms (Banuelos *et al.*, 1998). High intracellular [Na^+] is toxic to most organisms, including yeast (Banuelos *et al.*, 1998). In *S. cerevisiae*, high intracellular [K^+] and [Na^+] cause defects in growth (Banuelos *et al.*, 1998). Na^+ and K^+ extrusion are an important component of ionic homeostasis in cells (Banuelos *et al.*, 1998). In *S. cerevisiae*, two-thirds of the K^+ taken up by growing cells is returned to the external medium (Ortega & Rodriquez-Navarro, 1985). Na^+ and K^+ homeostasis in yeast is achieved by the coordinate activity of plasma membrane efflux and influx systems, K^+ channels (Lopez and Pena, 1999), and an efficient Na^+ sequestion system in a pre-vacuolar compartment (Rodriquez-Navarro and Ramos, 1984; Gaber, 1992; Rodriquez-Navarro *et al.*, 1994; Niu *et al.*, 1995; Serrano, 1996; Schachtman and Liu, 1999; Serrano *et al.*, 1999; Navarre and Goffeau, 2000).

Alterations in the Blm3p may cause increased levels of Na^+ and K^+ inside *blm3-1* cells, thereby altering osmotic regulation and causing defective growth. All cells in dealing with high concentrations of external solutes of any kind must make an osmotic adjustment (Brewester *et al.*, 1993; Maeda *et al.*, 1995; Hahnenberger *et al.*,

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1996). Osmotic adjustment of *blm3-1* cells after exposure to high concentrations of external solutes such as 2 M Na^+ and K^+ may be the reason why *blm3-1* cells display an increase in viability after these exposures (**Figure 3-11**).

An alteration in membrane integrity may also have an affect on the plasma membrane destabilizing activity of SDS, and be the reason why *blm3-1* cells displayed increased viability in the presence of 0.005% SDS (**Table 3-4**, **Figure 3-13**).

The fact that yeast has more than one type of efflux and influx system controlling intracellular $[Na^+]$ and $[K^+]$ (Navarre and Goffeau, 2000) strengthens the hypothesis that the *blm3Δ* cells may have a compensatory mechanism restoring alterations in growth and viability. Also, glucose uptake in *S. cerevisiae*, is mediated by transporters encoded by members of a large multigene family (Bisson *et al*., 1993; Kruckeberg, 1996). Glucose uptake is almost absent in cells in which *HXT1, HXT2, HXT3, HXT4, HXT6, HXT7* and *GAL2* are disrupted, and consequently the cells are unable to grow on glucose as a sole carbon source (Liang and Gaber, 1996). The reintroduction of any one of the disrupted *HXT* genes is able to restore strain growth on glucose (Liang and Gaber, 1996). The case for *BLM3* may be similar. It may be a member of a multigene family that when knocked out is able to be compensated for by other members of the family.

In the plasma membrane, MFS antiporter proteins can be driven by the PMF of the transmembrane electrochemical proton gradient ($\Delta\mu_{H+}$) rather than by ATP hydrolysis (Paulsen *et al*., 1993). The transmembrane proton gradient ($\Delta\mu_{H+}$) is composed of a chemical gradient of hydrogen ions (ΔpH) and electrical charge

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gradient ($\Delta\Psi$) (Paulsen *et al.*, 1993). Alterations in permeases can alter the plasma membrane potential affecting cellular function (Madrid *et al.*, 1998; Navarre and Goffeau, 2000). The plasma membrane potential is an important factor of toxic cation tolerance (Navarre and Goffeau, 2000). Alterations in Trk1p and Trk2p transporters of *S. cerevisiae* have been reported to affect membrane hyperpolarization (Madrid *et al.*, 1998) resulting in increased sensitivity to toxic cations, hygromycin B and trimethylammonium (TMA). Yeast plasma membranes contain a 55 amino acid hydrophobic polypeptide, Pmp3p, (Navarre and Goffeau, 2000). The *PMP3* gene is not essential under normal growth conditions. Deletion of *PMP3* increases plasma membrane potential and increases sensitivities to cytotoxic cations Na^+ and hygromycin B. These membrane potential defects have an effect on cation and drug uptake (Navarre and Goffeau, 2000). In *E. coli*, it has been suggested that the mechanism of bleomycin uptake is similar to that of aminoglycoside uptake, which may depend on the electrical potential across the inner membrane (Taber *et al.*, 1987; Collis and Grigg, 1989; Ichige and Walker, 1997). The case for *BLM3* may be similar. Alterations in *BLM3* cells may affect the plasma membrane potential, thereby increasing the uptake of cytotoxic cationic bleomycin (Burger, 1998) and K^+ and Na^+ cations.

b. Possible role of Blm3p as an MDR protein.

MDR proteins provide a cell with a means of defense against toxins of the environment (Lewis, 1994). MDR proteins fall into four recognized families, (Lewis, 1994), the ABC (ATP-binding cassette) family, the MFS family, the RND (resistance-nodulation-cell division) family, and the SMR (small multidrug

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resistance) family (Goffeau *et al.*, 1997). In yeast, several MDR pumps of the MFS have been identified (Goffeau *et al.*, 1997). The role of Blm3p as a MDR protein cannot be ruled out based on their increased resistance to a wide range of chemotheraputic agents (Goffeau *et al.*, 1997) . Testing a variety of chemotheraputic agents may confirm these results.

Although computer aided analysis suggest that Blm3p is a permease (Nelissen *et al.*, 1996), evidence supporting Blm3p as a transporter remains to be determined.

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Table 1. Strains and plasmids used

Strain or plasmid	Genotype[a] or selectable marker	Source
Saccharomyces cerevisiae		
CM1313-1B	*MATα ade2-40* or *ade2-2 trp1-1* or *trp5-12*	This laboratory
CM1313-1C	*MATα ade2-40* or *ade2-2 trp1-1* or *trp5-12*	This laboratory
CM1469-5A	*MATα ade2-40* or *ade2-1 ilvl-92 his3-11* or *his3-15 leu2-3* and/or *leu2-112 trp1-1* or *trp5-12*	This laboratory
CM1469-5B	*MATα leu2-3* and/or *leu2-112 ade2-40* or *ade2-1 trp1-1* or *trp5-12*	This laboratory
CM1469-5C	*MATa ade2-40* or *ade2-1 ura3-1 ilvl-92 trp1-1* or *trp5-12 ura3-1 blm3-1*	This laboratory
CM1469-6A	*MATα ade2-40* or *ade2-1 trp1-1 or 5-112 his3-11* or *his3-15 leu2-3* and/or *leu2-112 ura3-1blm3-1*	This laboratory
CM1469-8B	*MATa ade2-40* or *ade2-1 trpl-l* or *trp5-12 ilv1-92 his3-11* or *his3-15 ura3-1 blm3-1*	This laboratory
CM1522-9A	*MATa ura3-52 trp1Δ6 leu2Δ his3Δ200 Gal2*	This laboratory
CM1522-9B	*MATa ura3-52 trp1Δ63 leu2Δhis3Δ200 Gal2*	This laboratory
CM1522-9C	*MATα ura3-52 trp1Δ63 leu2Δ his3Δ200 Gal2*	This laboratory
CM1522-9D	*MATα ura3-52 trp1Δ63 leu2Δ his3Δ200 Gal2*	This laboratory
BMA1	*MATa/MATα ura3-52/ura-52 trp1Δ63/trp1Δ63 leu2Δ/leu2Δ his3Δ200/his3Δ200 Gal2/Gal2*	Baudin *et al.*, 1993
CM-1521	*MATa/MATα ura3-52/ura-52 trp1Δ63/trp1Δ63 leu2Δ/leu2Δ his3Δ200/his3Δ200 Gal2/Gal2 BLM3/blm3Δ::HIS3*	This laboratory
CM1522-9A containing *blm3::gfp-HIS3*	*MATa ura3-52 trp1Δ6 leu2Δ his3Δ200 Gal2 blm3::gfp-HIS3*	This laboratory

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Table 1. Strains and plasmids used (Continued)

Strain or plasmid	Genotype[a] or selectable marker	Source
CM-1522	*MATa/MATα ura3-52/ura-52 trp1Δ63/trp1Δ63 leu2Δ/leu2Δ his3Δ200/his3Δ200 Gal2/Gal2 BLM3/blm3Δ::HIS3*	This laboratory
CM-1523	*MATa/MATα ura3-52/ura-52 trp1Δ63/trp1Δ63 leu2Δ/leu2Δ his3Δ200/his3Δ200 Gal2/Gal2 BLM3/blm3Δ::HIS3*	This laboratory
CM-1524	*MATa/MATα ura3-52/ura-52 trp1Δ63/trp1Δ63 leu2Δ/leu2Δ his3Δ200/his3Δ200 Gal2/Gal2 BLM3/blm3Δ::HIS3*	This laboratory
CM-1525	*MATa/MATα ura3-52/ura-52 trp1Δ63/trp1Δ63 leu2Δ/leu2Δ his3Δ200/his3Δ200 Gal2/Gal2 BLM3/blm3Δ::HIS3*	This laboratory
Escherichia coli		
HB 101	*F⁻ mcrB mrr hsd*S20 (r_B^-, m_B^-) *rec*A13 *sup*E44 *ara*l4 *gal*K2 lacY1 *proA2 rpsL20(Smr) xyl5-leu mtl1*	Life Technologies (Gaithersburg, MD)
Plasmids		
YEp24	*amp tet 2μ URA3*	Botstein *et al.*, 1979
pRS415	*ARS CEN LEU2*	Stratagene (LaJolla, CA)
pAS128	*ARS CEN LEU2*	Strunnikov *et al.*, 1993
pPM311[b]	*amp tet 2μ URA3*	This laboratory
pRS303	pBluescript, *HIS3*	Sikorski and Hieter (1989)

[a]The genetic symbols used are described by Mortimer *et al.*, 1992.
[b]pPM311 complements the *blm3-1* mutation.

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Table 2. Primers for replacing *BLM3* gene with *HIS3*

Primer	Sequence (5' to 3')
Deletion primers[a]	
Oligopro	ATGATCTCAAACTGCTTCTTAATATAGGCATCCACCTTTTCTGGGACGCTTTTTA ctcttggcctcctctag
Oligoterm	CCAGTGGAGAAGTACACGATATTTGCAAAGTCTGTCATC AGGGCTTGATAATAAA tcgttcagaatgacacg
Test primers[b]	
Test primer 1	GGTGGCCGAGGTATCCCTTAG
Test primer 2	TGTTGAGGATTCAGGCCGACAACGGA

[a]Deletion primers contain 55 nucleotides of homology to the 5' flanking region (oligopro) and 55 nucleotides to the 3' flanking region (oligoterm) to the *BLM3* gene to be deleted (shown in upper case), and 17 nucleotides of *HIS3* sequence (shown in lower case).
[b]Test primers 1 and 2 from the flanking sequences of the *BLM3* ORF.

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Table 3. Primers for fusing GFP to the 3' end of *BLM3*

Primer	Sequence (5' to 3')
Primers[a]	
Forward	TTTGATCACCCATACGATCAGGTTCGCCAGCTGTCGCTAAACTAT **atgtctaaaggtgaagaa**
Reverse	TTCTGCTTCTAATAATGTGGTTGGATCTGAAATTGACGGATTACT **gcgcgcctcgttcagaatg**
Test primers[b]	
Forward	TATGTACGTCATGTCGCCGAAAGC
Reverse	GCAATCCCGCAGTCTTCAGTGGTG

[a]Forward primer contains the last 45 bases of the *BLM3* coding sequence, excluding the stop codon (shown in upper case) and the first 18 bases of the GFP coding sequence (shown in lower case). Reverse primer contains 45 bases of homology downstream from the 3' end of *BLM3* (shown in upper case) (about 20 nucleotides away from the 3' end of *BLM3*) and 18 bases in the *HIS3* untranslated region (shown in lower case). [b]Forward test primer (20 bases long) about 680 bases upstream from the end of the *BLM3* gene and a reverse test primer (20 bases long) in the *HIS3* gene.

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Table 3-1. 2:2 Co-segregation of *HIS3* marker and phleomycin hypersensitivity

[a]Segregant (CM-1522)	[b]*HIS3*$^+$/*HIS3*$^-$	[c]Score	[d]Phleomycin [e](R/S)
1A	*HIS3*$^-$	4.0	R
1B	*HIS3*$^+$	3.0	S
1C	*HIS3*$^+$	3.0	S
2A	*HIS3*$^+$	3.0	S
2B	*HIS3*$^+$	3.0	S
2C	*HIS3*$^-$	4.0	R
3A	*HIS3*$^+$	2.5	S
3B	*HIS3*$^-$	4.0	R
3C	*HIS3*$^+$	2.5	S
3D	*HIS3*$^-$	4.0	R
4A	*HIS3*$^+$	1.5	S
4B	*HIS3*$^-$	4.0	R
4C	*HIS3*$^+$	2.5	S
4D	*HIS3*$^-$	4.0	R
5A	*HIS3*$^-$	4.0	R
5B	*HIS3*$^+$	3.5	S
5C	*HIS3*$^+$	3.5	S
5D	*HIS3*$^-$	4.0	R
6A	*HIS3*$^-$	4.0	R
6B	*HIS3*$^+$	3.0	S
6C	*HIS3*$^-$	4.0	R
7A	*HIS3*$^-$	4.0	R
7B	*HIS3*$^+$	2.5	S
7C	*HIS3*$^+$	2.5	S
7D	*HIS3*$^-$	4.0	R
8A	*HIS3*$^-$	4.0	R
8B	*HIS3*$^+$	3.0	S
8C	*HIS3*$^-$	4.0	R
9A	*HIS3*$^-$	4.0	R
9B	*HIS3*$^+$	2.5	S
9C	*HIS3*$^+$	2.5	S
9D	*HIS3*$^-$	4.0	R
10A	*HIS3*$^-$	3.5	R
10B	*HIS3*$^+$	3.0	S
10C	*HIS3*$^-$	4.0	R
10D	*HIS3*$^+$	3.0	S

[a]Tetrads dissected from diploid CM-1522
[b]Segregants tested on SD+7-his
[c]Score of resistance and sensitivity
[d]Segregants tested on SD+7-his +7μg/ml phleomycin
[e]R: Resistance S: Sensitivity

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Table 3-2. Growth of *BLM3* and mutant *blm3-1* and *blm3Δ* strains on SD + Calcofluor

[a]Strain	Cells/plate 0 μg/ml	Cells/plate 20 μg/ml	Cells/plate 80 μg/ml
CM1469-5A *BLM3*	32 ± 0.33	26 ± 1.1	44 ± 6.0
CM1469-2A *blm3-1*	48 ± 3.5	39 ± 0.50	42 ± 3.4
CM1469-5C *blm3-1*	41 ± 4.9	26 ± 3.3	39 ± 3.0
CM1522-9A *BLM3*	62 ± 6.1	53 ± 5.6	63 ± 2.7
CM1522-9B *blm3Δ*	64 ± 6.0	60 ± 5.2	63 ± 4.9

***BLM3* and mutant *blm3-1* and *blm3Δ* strains were grown in liquid supplemented SD medium and plated at $5x10^6$ cells/ml on complete SD medium containing 0, 20 and 80 μg/ml of calcofluor at 30°C for 3 days (100 cells/plate on three separate plates).**

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Table 3-3. Expression or lack of expression of the 5.4 kb *BLM3* transcript in human tissues.

High Expression[a]	Low Expression[a]	No Evidence of Expression[b]	
adrenal gland	occipital lobe	amygdala	fetal brain
aorta	thalamus	appendix	fetal heart
bladder	pancreas	cerebellum	fetal kidney
bone marrow		cerebral cortex	fetal liver
caudate nucleus		frontal lobe	fetal spleen
colon		hippocampus	fetal thymus
heart		kidney	
mammary gland		liver	
peripheral leukocyte		lung	
pituitary gland		medulla oblongata	
putamen		ovary	
skeletal muscle		placenta	
small intestine		prostate	
spleen		salivary gland	
substantia nigra		spinal cord	
subthalamic		stomach	
nucleus		testis	
temporal lobe		trachea	
thyroid gland		whole brain	
thymus			
uterus			

[a]High or low expression of the 5.4 kb *BLM3* transcript in adult human tissues.
[b]No evidence of expression of the 5.4 kb *BLM3* transcript in adult and fetal human tissues.

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Table 3-4 Liquid Assays for growth with and without 0.005% SDS[a]

Strain	Growth YNB-2% glc	Growth YNB-2% glc+0.005% SDS
CM1469-2A ***blm3-1***	++	++
CM1469-5A ***BLM3***	++	+/-
CM1469-5C ***blm3-1***	++	++

[a]Strains were grown from a starting density of 5 x 10^5cells/ml for 2 days at 30°C in liquid YNB-2% glc and YNB-2% glc+0.005% SDS. and checked for growth.

++ **growth**
+/- **very little growth**

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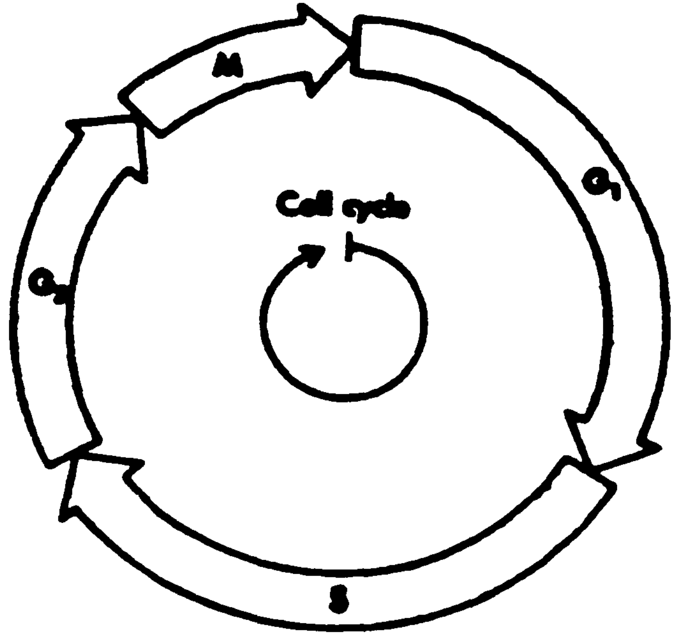

Figure 1. Phases in the life cycle of a yeast cell (Henschke, P.A. and Rose, A.H., 1987). M = mitosis, G1 = pre DNA synthesis, S = DNA synthesis, G2 = period between DNA synthesis and mitosis (Howard and Pelc, 1953). Checkpoints occur at the G1/S, within S and G2/M stages (Hartwell and Kastan, 1994).

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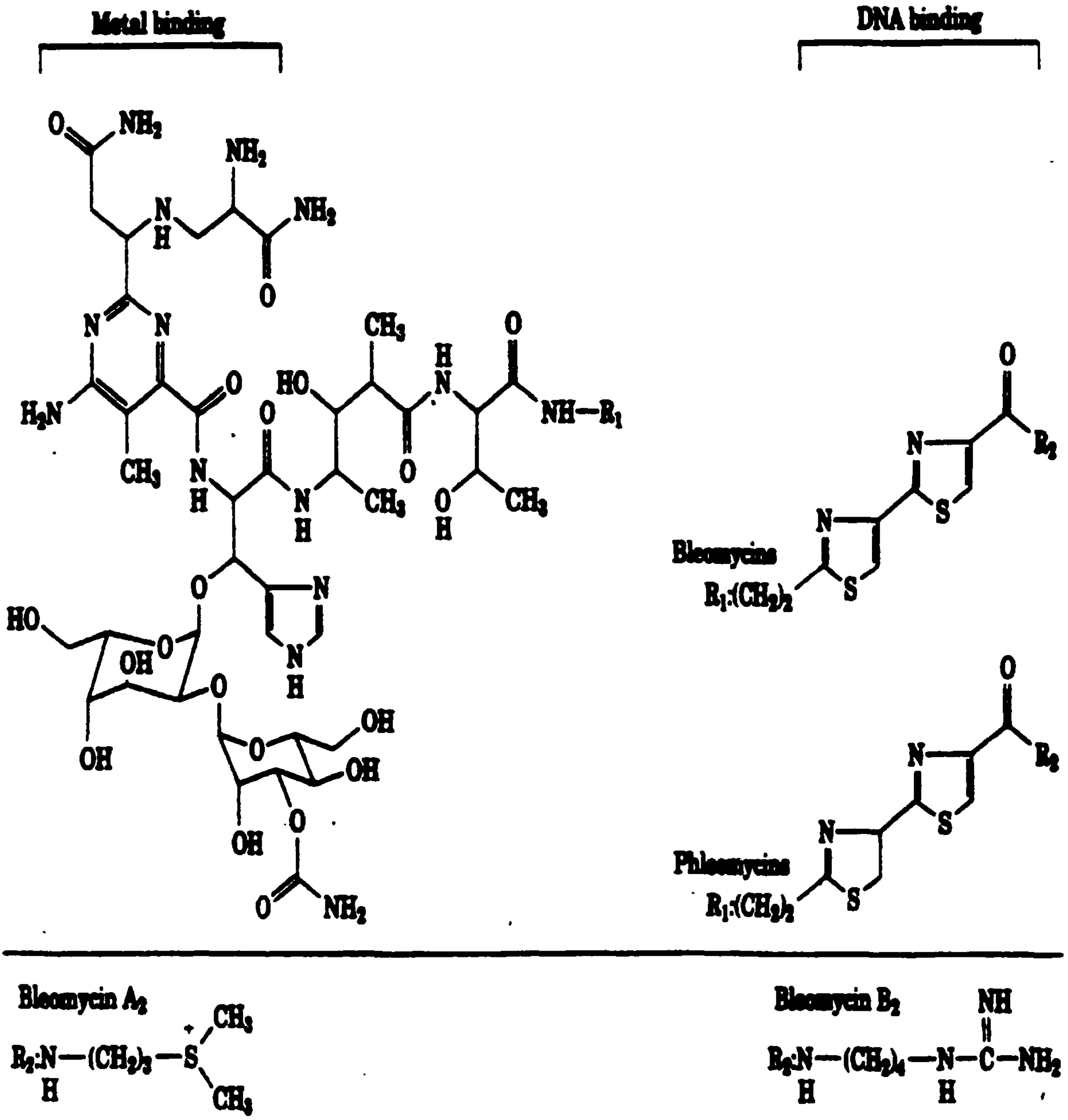

Figure 2. Structures of bleomycins and phleomycins (Takita *et al.*, 1972a 1972b, 1978; Naganawa *et al.*, 1977). Figure taken from Moore (1999).

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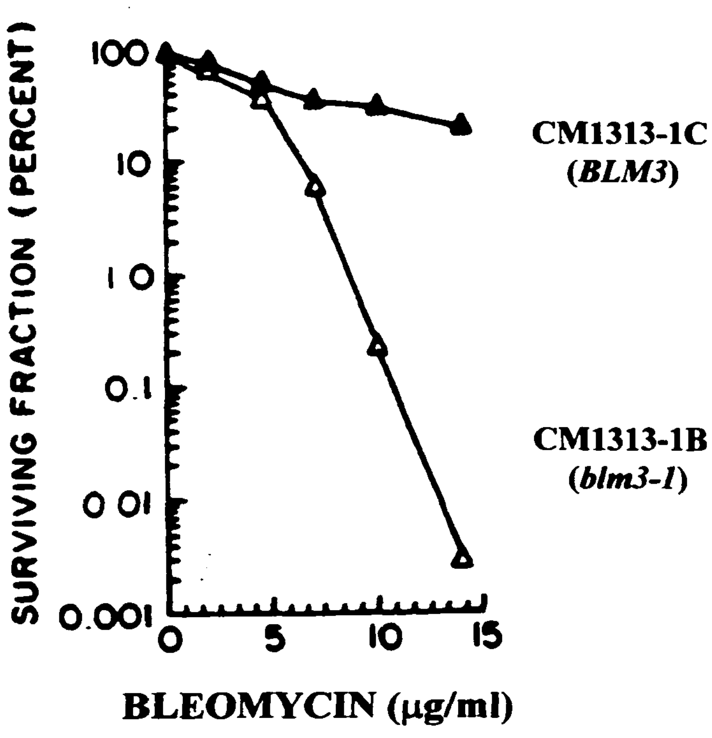

Figure 3. Survival of *BLM3* (CM1313-1C) cells and *blm3-1* (CM1313-1B) mutant cells (unpublished data were provided by Dr. Moore and printed with her permission). Cells were plated and grown on YPAD containing (0, 2.5, 5, 7.5, 10 and 15 μg/ml) at 30°C as described in Moore (1991).

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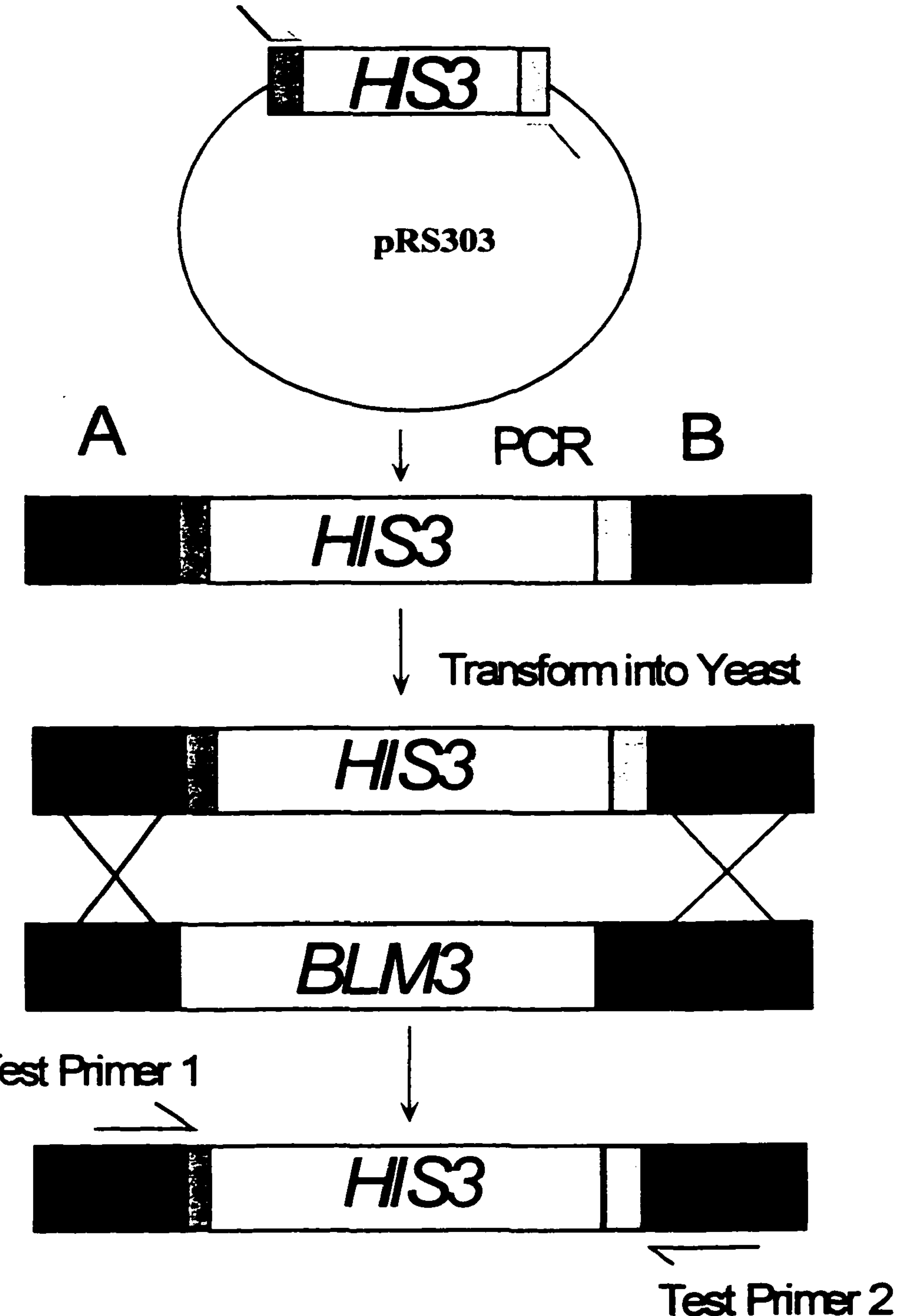

Figure 4. Deletion of the chromosomal *BLM3* gene. 1) PCR amplification of gene replacement *HIS3* cassette; (2) transformation of yeast with PCR product; (3) verification by PCR of the correct gene deletion using test primers.

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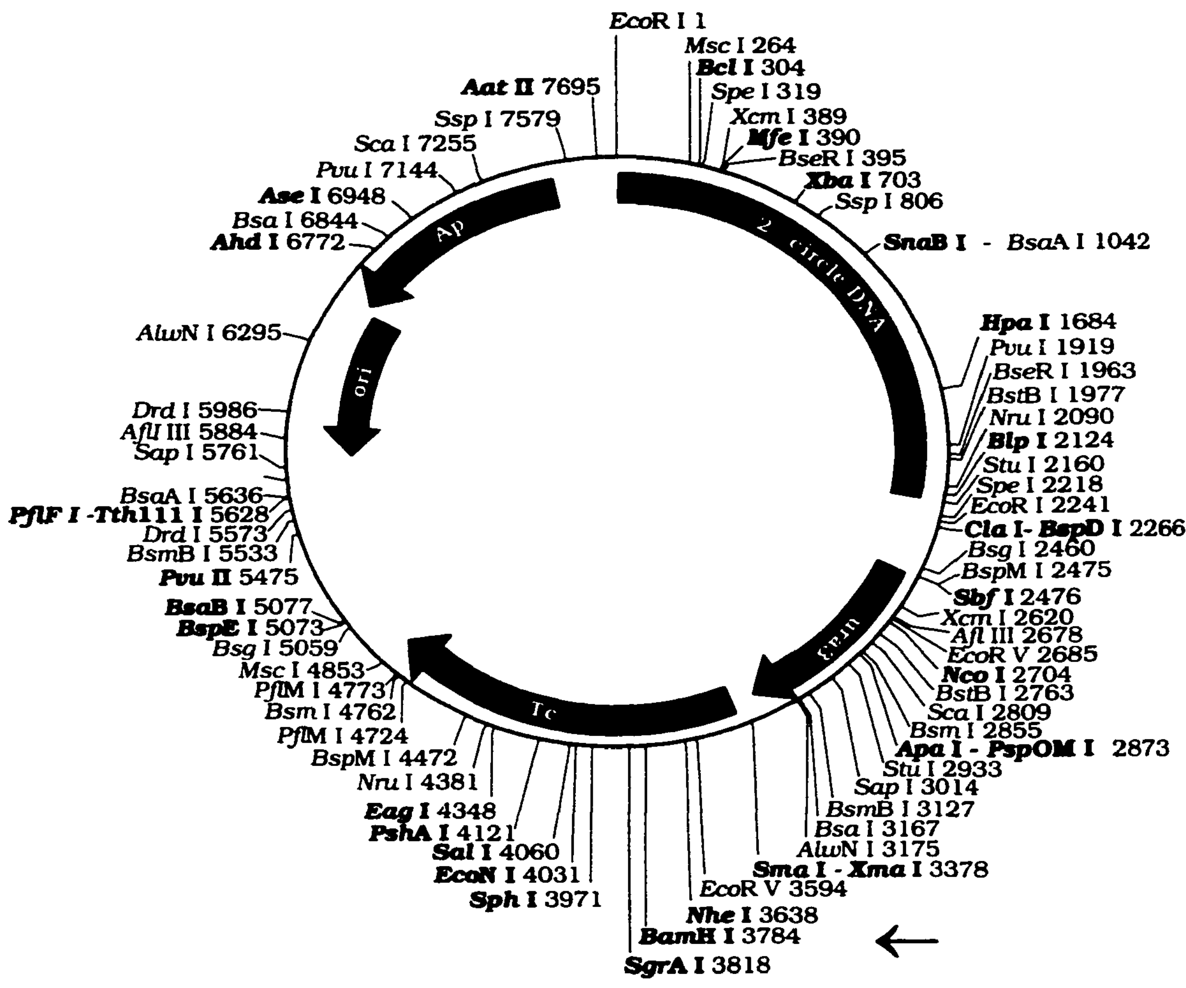

Figure 1-1. Multicopy plasmid shuttle vector, YEp24, and restriction map (New England Bio Labs, MA, 2000). The DNA insert complementing *blm3-1* had been inserted at the *Bam*HI site (←) within the tetracycline gene (Tc). The unique sites are shown in bold type (Bothstein *et al.*, 1979).

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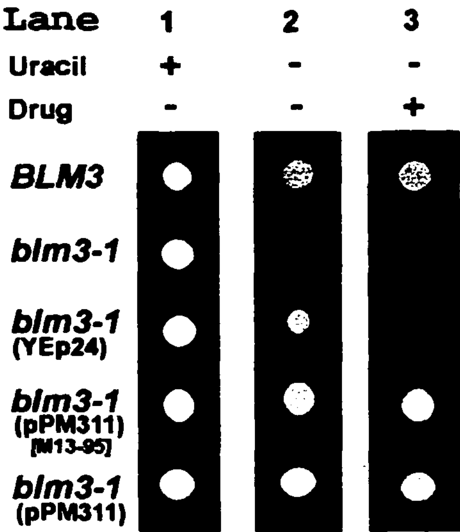

Figure 1-2. Complementation of the *blm3-1* mutation. Growth of strains on the synthetic complete medium (SD+7, lane 1), SD+7 lacking uracil (-ura; lane 2), and SD+7- ura + 7μg/ml of phleomycin (lane 3) are illustrated. Row 1: The *BLM3* control strain (CM1469-5B) grew on all three media. Row 2: The *blm3-1* mutant strain(CM1469-8B) is auxotrophic for uracil and thus grew on synthetic complete medium, but did not grow on SD+7-ura or SD+7-ura+ 7μg/ml. Row 3: The *blm3-1* mutant (CM1469-8B) transformed with the YEp24 vector permitted growth on media lacking uracil and drug. Row 4: The (CM1469-8B) transformant (M13-95) isolated from yeast containing pPM311 grew on SD+7- ura +7μg/ml. Row 5: The plasmid pPM311 isolated from *E. coli* also complemented the *blm3-1* mutation on SD+7-ura+ 7μg/ml (CM1469-5C [pPM311]).

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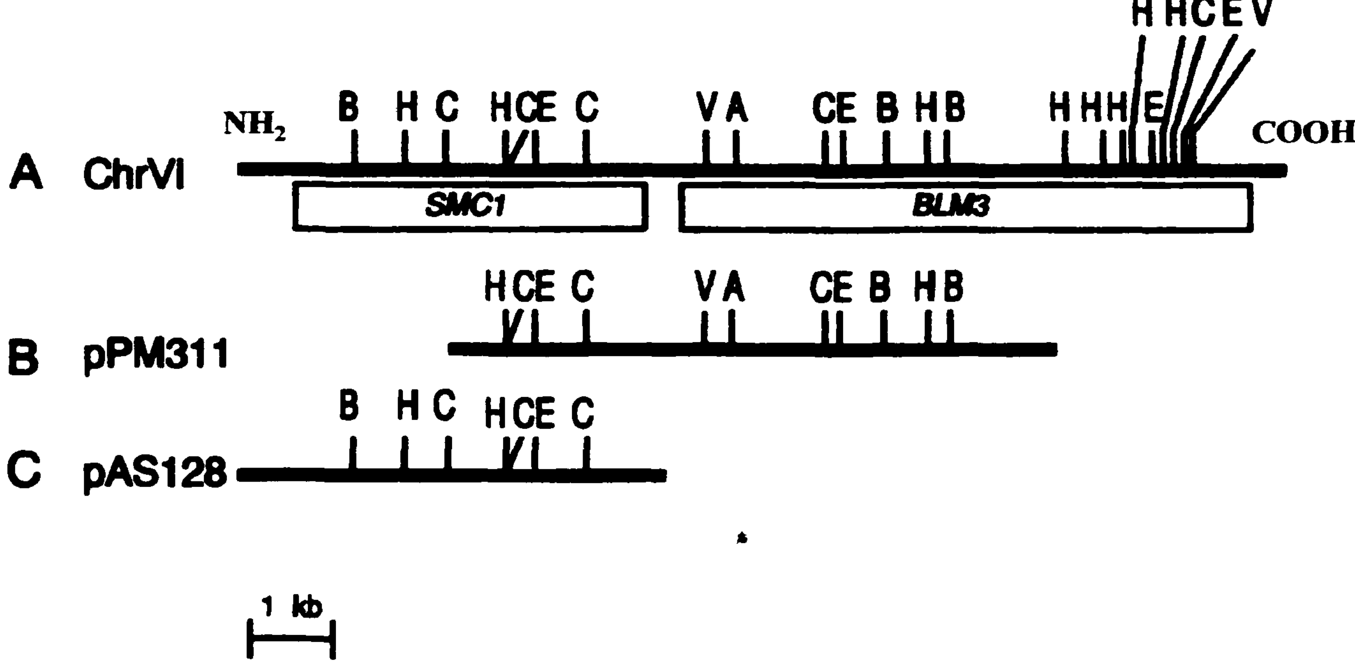

Figure 2-1. Region of chromosome VI which has been studied. A) Restriction map of genomic DNA: A: *ApaI*, B: *Bam*HI, C: *ClaI*E: *Eco*RI, H: *Hind* III, V: *Eco*RV. B) The restriction map of the cloned insert from pPM311 which complemented the *blm3-1* mutation. The insert encompassed part of the *SMC1* gene and 57% of the *BLM3* gene. C) The insert from pAS128 bearing the *SMC1* gene.

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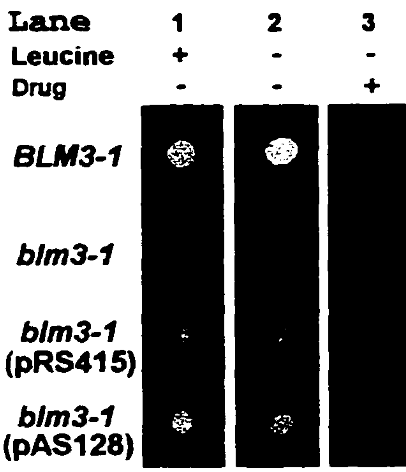

Figure 2-2. The *SMC1* gene does not complement the *blm3-1* mutation. Growth of strains on synthetic complete medium (SD+7, lane 1), SD+7 lacking leucine (-leu: lane 2) and SD+7-leu+ 7μg/ml of phleomycin (lane 3) are illustrated. Row 1: The *BLM3* control strain (CM1469-5B) grew on all three media. Row 2: The *blm3-1* mutant strain (CM1469-6A) is auxotrophic for leucine and thus grew on synthetic complete medium, but did not grow on SD+7-leu or SD+7-leu+ 7μg/ml. Row 3: The *blm3-1* mutant (CM1469-6A) transformed with pRS415 (vector only) grew on SD+7 and SD+7-leu. Row 4: The (CM1469-6A) transformant containing pAS128 (*SMC1*) did not grow on SD+7-leu+ 7μg/ml, comparable to the *blm3-1* mutant (CM1469-6A) transformed with pRS415 (row 3).

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A

```
      ATGACCGCTAACAATGACGATGATATCAAATCACCCATTCCCATTACTAACAAGACCTTA    60
 20   *M  T  A  N  N  D  D  D  I  K  S  P  I  P  I  T  N  K  T  L

      TCCCAATTGAAGCGCTTTGAGAGAAGTCCAGGAAGGCCCAGTTCTTCTCAGGGCGAGATA   120
 40   S  Q  L  K  R  F  E  R  S  P  G  R  P  S  S  S  Q  G  E  I

      AAACGTAAAAAGTCTAGGCTATATGCCGCAGACGGAAGACCACATTCTCCGCTAAGAGCA   180
 60   K  R  K  K  S  R  L  Y  A  A  D  G  R  P  H  S  P  L  R  A

      AGGTCTGCTACCCCAACGCTACAGGACCAAAAACTGTTCAATGGCATGGATTCCACTTCC   240
 80   R  S  A  T  P  T  L  Q  D  Q  K  L  F  N  G  M  D  S  T  S

      CTTTTGAATGAAAGGCTACAGCATTATACGCTGGATTATGTTAGTGACAGGGCCCAGCAT   300
100   L  L  N  E  R  L  Q  H  Y  T  L  D  Y  V  S  D  R  A  Q  H

      ATGAAGAATATATATGACCCATCATCTCGCTGGTTCAGCAGATCCGTGAGGCCTGAATTT   360
120   M  K  N  I  Y  D  P  S  S  R  W  F  S  R  S  V  R  P  E  F

      CCTATTGAAGAGTTCTTACCATATAAGACTGAAAGTCATGAAGATCAAGCGAAATACTTG   420
140   P  I  E  E  F  L  P  Y  K  T  E  S  H  E  D  Q  A  K  Y [1]L

      TGCCATGTCTTAGTTAATCTTTATATTGCGATCAGCTCATTAGATATACAAGGTTTGATT   480
160   C  H  V  L  V  N  L  Y  I  A  I  S  S  L  D  I  Q  G  L  I

      TCTATTTCCAGTAAAGATCTGGCTGATTTAAAGAAAGAAGTGGATGATTTAGCTCTTAAA   540
180   S  I  S  S  K  D  L  A  D  L  K  K  E  V  D  D  L  A  L  K

      ACCGATCTTTTCAGGTTATCTAACAACACAGCGGAGAATGACTTACTTGGTAACGATATT   600
200   T  D  L  F  R  L  S  N  N  T  A  E  N  D  L  L  G  N  D  I

      GCTGATTATGACGATGCGGAAGGCCTGGAGGACGAATTGGATGAATACTTCGACTTAGCA   660
220   A  D  Y  D  D  A  E  G  L  E  D  E  L  D  E  Y  F  D  L  A

      GGCCCCGACTTTAATGCTACCGGAAAAATCACAGCTAAATCAGCTACTATTGTGAATGTA   720
240   G  P  D  F  N  A  T  G  K  I  T  A  K  S  A  T  I  V  N  V

      AACCATTGGACTAATGAGCTCAAAAATTGTCTACATTTTGATTTTCCAGTAGCTTTAAGA   780
260   N  H  W  T  N  E  L  K  N  C  L  H  F  D  F  P  V  A  L  R

      AAGTCGCTAGCGACAGTTTATTATTATTTGTCTCTTGTTCAGGGCCAAAAGGTGTATAGA   840
280   K  S  L  A  T  V  Y  Y  Y  L  S  L  V  Q  G  Q  K  V  Y  R

      CAAATGCATGTCGATATGTTCGAAAGATTAGTAAGCCTTGACGATGATAGGACAAATTTC   900
300   Q  M  H  V  D  M  F  E  R  L  V  S  L  D  D  D  R  T  N  F

      ACTGAACTGTTGCAAAAACAAGGTCTTTTGCTAGATCATCAAATCATGCTCAATTTCCTG   960
320   T  E  L  L Q  K  Q  G  L  L  L  D  H  Q  I  M  L  N  F  L

      TGCGAATTTCTACCTTATCCAGATCCCGACTATGCTCGTTATGAATTATCATCAAAGGA  1020
340   C  E  F  L  P  Y  P  D  P  D  Y  A  R  Y  E  L  S  S  K  E

      GATTTACAGTTATTTAGATTACTTTTGAAGCATGCACATAATGCAAAACCATTTTTCGAT  1080
360   D  L  Q  L  F  R  L  L  L  K  H  A  H  N  A  K  P  F  F  D

      AAGTCAAAGGAAAGTTTGTTAGTTGATACGATGAATTTTCTGTTGTCTAGTCTTGCACCA  1140
380   K  S  K  E  S  L  L  V  D  T  M  N  F  L  L  S  S [2]L  A  P

      TCTACTATGATGGCTGTCATGCCTATCGTTACATCCGTTGTGCCCTATCATTATCATATC  1200
400   S  T  M  M  A  V  M  P  I  V  T  S  V  V  P  Y  H  Y  H  I

      CATTCTAAGATCATCGATTATTTCCCGTTTTGCTATAGCATCTGGAGCTCAGTCAGCGCA  1260
420   H  S  K [8]I  I  D  Y  F  P  F  C  Y  S  I  W  S  S  V  S  A

      AACGTGGCCATCGACACTCACATGTATGATTTTGTTGGGTCAATTTCCAAAGACGTTCAT  1320
440   N  V  A  I  D  T  H  M  Y  D  F  V  G  S  I  S  K  D  V  H
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460   N K I L S S E H E K D [9]V V G V E F G E F

      GGGATTTTTACTGATGATCAAATGACTTTTATGTTCAATAGGTTACAAGGCCATCTTAGA  1440
480   G I F T D D Q M T F M F N R L Q G H L R

      ACAGACGGTCAAATACATTCGTATTCCCGCACAGTGAAGCCTTTTGTTTATGCTATAAAC  1500
500   T D G Q I H S Y S R T V K P F V Y A I N

      GGATCAAAAAAAGATAGGTTTTTTGAAAAACTTGTAAGTTTAGCCAAAGCAATCGAAACA  1560
520   G S K K D R F F E K L V S L A K A I E T

      TTTATCCATCCCTCTAATAATGGGTTTTGGACCAAGCCAAATGCTAAATTCGTTCATGCA  1620
540   F I H P S N N G F W T K P N A K F V H A

      TTTATAAAGTCTTACCACGGAAGGGTCAAATATGAAGAAGATATTTGTGCTAGGGGCGTC  1680
560   F I K S Y H G R V K Y E E D I C A R G V

      ACAAATGGGATATGTTTAACTTCTTTTTGTCACGAAGAGATAGTTGAAATATTCTTAAAT  1740
580   T N G [10]I C L T S F C H E E I V E I F L N

      ATTATCAGTCTGGGTTCACAGAATAAAAATCCTGATATTGCGAACTATTACATCTCTTGT  1800
600   I I S L G S Q N K N P D I A N Y Y I S C

      TTCGCATATCTGTTAGAGCTGGATCCTTCAAATGCATATTTAATTTATGACAAAATACTG  1860
620   F A Y L L E L D P S N A Y L I Y D K I L

      ATAGATTTGTACGATACGCTGGCTGACCAATTTATCAATTCGAGACACAGAATCATTTCC  1920
640   I D L Y D T L A D Q F I N S R H R I I S

      TCTTTGAAACAATTTACAAGAGTAATTCGGTTTATTGTGATGGATAAGCTATATCGCGTG  1980
660   S L K Q F T R V I R F I V M D K L Y R V

      CACATAACAAACGTCCTTTCGATGCTGGTCTCCAAACTTGATATGAACGATACTAATTTG  2040
680   H I T N V L S M L V S K L D M N D T N L

      ACAAGCAACCTCATCAACGGTATTGTATCTATAGCCGCTTTCATTCCTATCCAAGACCTC  2100
700   T S N [3]L I N G I V S I A A F I P I Q D L

      ACTGGGGAGGACGATTATATATCGTTTGAATCGGATACTCTTCCTTTAGTTCAACAACAT  2160
720   T G E D D Y I S F E S D T L P L V Q Q H

      TTTTATCATATTAAGTGTGGCGAAAGTTCAAAGACCTTCCGAGTTGATGATGAACTGTTA  2220
740   F Y H I K C G E S S K T F R V D D E L L

      AATAACGCTTTCAAAGCTTCCACTACAGTTTTTCAAAGTATGCTAAAAGTATACGTAGAA  2280
760   N N A F K A S T T V F Q S M L K V Y V E

      AAAATTTTCCAATTGGTTGATGTAGACTTAGAGGACTCTTTGGTTACTAAAATAAACCAA  2340
780   K I F Q L V D V D L E D S L V T K I N Q

      ACAACTATGATTTTACAAGAGTCTATGGACGATAAAATATTTAATTATTTTGCTTCTTTA  2400
800   T T M I L Q E S M D D K I F N Y F A S L

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820   L Q R N F W S N D S F K E K D P N Y E L

      GTTACTATCCCACTAGCGGCTTTAGTAAGAAGGAATAATGGTTTGAGTAAAGAGTTGGTC  2520
840   V T I P L A A L V R R N N G L S K E L V

      AGAACTCTTTTATTCCATATCAAAGAACAAATCAAAAGAGGCGCCGGGTCTGTAAGAAGT  2580
860   R T L L F H I K E Q I K R G A G S V R S

      ACTTCAGAGATTCAACAGAGGGATGTTAAGTTAGTTTTATACTTAACTGCACTAAATGAT  2640
880   T S E I Q Q R D V K L V L Y L T A L N D

      GTCTTAAGGCAATGTCATGAATCTCTATTGGAGTATAGCGATGAGTTGATAACATTCATG  2700
900   V L R Q C H E S L L E Y S D E L I T F M

      AAATATTTATACGACAACGTCACTAACCCGCCATTGGATGTTATCACATCTATTGTTATT  2760
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 940   H  S  A  L  A  T  L  C  T  T  E  I  T  D  C  R  L  F  P  E

       GACTCTAAGATTCCGGAAAAAGACAGATGGGGAGGACTACAGTTCGATCCTCGAAGATTT  2880
 960   D  S  K  I  P  E  K  D  R  W  G  G  L  Q  F  D  P  R  R  F

       GATAAACAGCATTTGAGTTTTCAGTGGCACGTACCTTCTAGTGACGAGATAACTTTATCC  2940
 980   D  K  Q  H  L  S  F  Q  W  H  V  P  S  S  D  E  I  T  L  S

       ATAAGCATTCTAGAAAGCCTTTCCGAATACTGTATTAACAACGTAGAAGAACTGATGAAA  3000
1000   I  S  I  L  E  S  L  S  E  Y  C  I  N  N  V  E  E  L  M  K

       GCTCCAAGACATGATTCCGAATATGGCGATATGATACAAAAATATGTTTTAGTTATGACA  3060
1020   A  P  R  H  D  S  E  Y  G  D  M  I  Q  K  Y  V  L  V  M  T

       CATACGCTTTCCGGGTCAAGTTTACTTTTTGATCCAGATTTTAACAAATATAGGACGCAA  3120
1040   H  T  L  S  G  S  S  L  L  F  D  P  D  F  N  K  Y  R  T  Q

       TCAAACTTATCATACAGAGAGAAACTGATTTTATTAAAGAATATACGCGAAAATAATTGT  3180
1060   S  N  L  S  Y  R  E  K  L  I  L  L  K  N  I  R  E  N  N  C

       GACCCTCAAGAACTGGATATTGATATTGAACAAATTCGTTCTGGCAAGGATGATGAAGAC  3240
1080   D  P  Q  E  L  D  I  D  I  E  Q  I  R  S  G  K  D  D  E  D

       TATATTGAGAGTAAGGACATTGAAGCAGGGCTGAATGCAGGAGTTTCCGATGTTGTGCAG  3300
1100   Y  I  E  S  K  D  I  E  A  G  L  N  A  G  V  S  D  V  V  Q

       TTAAGAGATGAGTTTCCGGATGAATTAATTGTTGATGAAGAAGTAGTGTCTGAGATGCCA  3360
1120   L  R  D  E  F  P  D  E  L  I  V  D  E  E  V  V  S  E  M  P

       TCTGGTGTAAATACCCCTATCGCGGGGACGCATGGCACGGACAATTCAGCTATGAGTTCG  3420
1140   S  G  V  N  T  P  I  A  G  T  H  G  T  D  N  S  A  M  S  S

       GATCTAGCTTTCAGGGATTTAGATATTTACACCTGTAATTATTACTTCGGAAATACCACT  3480
1160   D  L  A  F  R  D  L  D  I  Y  T  C  N  Y  Y  F  G  N  T  T

       GAGGAGAAGTTACAAAACCCACAATATTTACAAGTCCACAGAGTTAGAGCGCGCATTGGA  3540
1180   E  E  K  L  Q  N  P  Q  Y  L  Q  V  H  R  V  R  A  R  I  G

       CATTTCTTTCACAAACTCTATGTTTTTTTATCTACAAACTTCGAAAACAACACCAACATG  3600
1200   H  F  F  H  K  L  Y  V  F  L  S  T  N  F  E  N  N  T  N  M

       TTCCAAATTCTATTGCACGGATTGAAAGTTTGGTTCACAGATCTGGGACAAGAAACGGTC  3660
1220   F  Q  I  L  L  H  G  L  K  V  W  F  T  D  L  G  Q  E  T  V

       TTCAATGAAGACCCAAATGCCTTCATTGACGTTGATTTCCTAGAAAACGTTCAATCTCTC  3720
1240   F  N  E  D  P  N  A  F  I  D  V  D  F  L  E  N  V  Q  S  L

       TCACACGTAAATGAGCCCTTCACGAGAACCAATTTTGCAATCAGAGCAAACAGTTTGCAC  3780
1260   S  H  V  N  E  P  F  T  R  T  N  F  A  I  R  A  N  S  L  H

       CAGAGTAGAGTTCTATTACATTCAACAAATAGAAAAGCTTCCAAGCTGGAAAACCTATTG  3840
1280   Q  S  R  V  L  L  H  S  T  N  R  K  A  S  K  L  E  N  L  L

       TTAGTTGACATCATACAGTTAGCGACATCCCTTTATCCTGATATTTATAAACCAGCACAG  3900
1300   L  V  D  I  I  Q  L  A  T  S  L  Y  P  D  I  Y  K  P  A  Q

       GGAACTTTGGTACACTGTATGAAACAATTAGTTGGGTCATATGGCGTAGTCATCAATAAA  3960
1320   G  T  L  V  H  C  M  K  Q  L  V  G  S  Y  G  V  V  I  N  K

       ATTATTCCATCATTAGAGAAAGCGATTAAGGATCATGATTATATGAAAATCCAAGTTATT  4020
1340   I  I  P  S  L  E  K  A  I  K  D  H  D  Y  M  K  I  Q  V  I

       TTAAATGTTTTGTTAATTAAGAAAATCCATAGGAAGCTTATGACGGATTATAAAGACATC  4080
1360   L  N  V  L  L  I  K  K  I  H  R  K  L  M  T  D  Y  K  D  I

       GGCAGATTGATATTTCTGCTTATTGAATGTTGTCGTGTGAATGAATTAGAGATTGGTATG  4140
1380   G  R  L  I  F  L  L  I  E  C  C  R  V  N  E  L  E  I  G  M

       TATGCAGATAAAATCTTAACTGACATAGTGATTGGGATCAAGATTCCTTCTAGTGTATGT  4200
1400   Y  A  D  K [5]I  L  T  D  I  V  I  G  I  K  I  P  S  S  V  C
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1420   V  I  S  D  Q  A  F  L  P  L  A  P  P  D  G  T  I  N  L  Q

      GTTGAAGCGGTAAAGCTTGCCAAAAAGAAAAAGCGTGAGTACTACCTCTCTCTGTTAGTG  4320
1440   V  E  A  V  K  L  A  K  K  K  K  R  E  Y  Y  L  S  L  L  V

      GATTTGCAGGACAAACTTTTAGACAAATTAGATAATGAAAAAGATATGGGGTGGAAGATA  4380
1460   D  L  Q  D  K  L  L  D  K  L  D  N  E  K  D  M  G  W  K  I

      AGAATGTTCATTTTACGTTTTGTTACACAAATCCAATCGAACCTCGAAAGCAAACCCGAT  4440
1480   R  M  F  I  L  R  F  V  T  Q  I  Q  S  N  L  E  S  K  P  D

      AAAAGAGCAGTATTTTCAATAATCTCCCAAATCTCCACAAAACATCCCGAAATCATACAT  4500
1500   K  R  A  V  F  S  I  I  S  Q  I  S  T  K  H  P  E  I  I  H

      TTGGTTGTAAAGTCATTGTTGTCGACGTGCAACAAGATAATATCTCTCTCTGACTATGAA  4560
1520   L  V  V  K  S  L  L  S  T  C  N  K  I  I  S  L  S  D  Y  E

      TATGACATCACCAGGGCCTATAAGAATGAATTCAATCCATCATTTGTGGAAATACTGGAC  4620
1540   Y  D  I  T  R  A  Y  K  N  E  F  N  P  S  F  V  E  I  L  D

      ACTTCGACCACAAGCTTCCCTAAAACGTTTACTGAAGAAATGAATAACTTTGATAACCCC  4680
1560   T  S  T  T  S  F  P  K  T  F  T  E  E  M  N  N  F  D  N  P

      AAGTATTTTATCGATTTGAGGGCGTATGTAGGGTGGCTATGTTGGGGAAGGCTTATGTAC  4740
1580   K  Y  F  I  D  L  R  A  Y  V  G  W  L  C  W  G  R  L  M  Y

      GTCATGTCGCCGAAAGCTTTAAAGCTCAATTTACGTGAGAATGAACTGGAAGTCCTCAAG  4800
1600   V  M  S  P  K  A  L  K  L  N  L  R  E  N  E  L  E  V  L  K

      ACAGCTGGTCATCTATTGACAAGAGAATTCCTGAGAGATGTTACAATGAATTTAGTCCAA  4860
1620   T  A  G  H  L  L  T  R  E  F  L  R  D  V  T  M  N  L  V  Q

      GATAATGAAACTAGGGGTGTTTTTAGTAGTGGTAACGTGTCATTTTTCTCTTTAGTAATC  4920
1640   D  N  E  T  R  G [6]V  F  S  S  G  N  V  S  F  F  S  L  V  I

      CTTTTGATATCATCTGGTTTCTGCGAACTGAATATGTCGGATCTCTTTGAGCTATGTGAA  4980
1660   L  L  I  S  S  G  F  C  E  L  N  M  S  D  L  F  E  L  C  E

      TCCTACTATAACAAAGACGATAAGGCTTCGATGATCATGTCTGTCGAGATAGTGGCTGGC  5040
1680   S  Y  Y  N  K  D  D  K [7]A  S  M  I  M  S  V  E  I  V  A  G

      TTAGTTTGCGGGAGTAAGTTTATGTCAGTCTCTGACTTGGACAAACGTGACACTTTTATC  5100
1700   L  V  C  G  S  K  F  M  S  V  S  D  L  D  K  R  D  T  F  I

      GAAAACTTCCTAGCCAAATGTTTAGATTATGAGTTGAACCATGACGCATTTGAAATTTGG  5160
1720   E  N  F  L  A  K  C  L  D  Y  E  L  N  H  D  A  F  E  I  W

      AGCACCTTGGCATGGTGGTTGCCTGCAGTCGTTGATTTAAGAAGGTCTAAAACTTTTTTT  5220
1740   S  T  L  A  W  W  L  P  A  V  V  D  L  R  R  S  K  T  F  F

      TGCCATTTTATCAACGCCGATGGCATGTTTGACCGTGAATCTGATGCAGCCACACATCAA  5280
1760   C  H  F  I  N  A  D  G  M  F  D  R  E  S  D  A  A  T  H  Q

      ACCTCCAAAATTTACATGCTAAGAAGTATCTTGATGAGCATGGAATTTAGAGCCCCAGAT  5340
1780   T  S  K  I  Y  M  L  R  S  I  L  M  S  M  E  F  R  A  P  D

      GTTGGTAAGCTATTTGATGAGTTGGTATTTGATCACCCATACGATCAGGTTCGCCAGCTG  5400
1800   V  G  K  L  F  D  E  L  V  F  D  H  P  Y  D  Q  V  R  Q  L

      TCGCTAAACTATTGA                                               5415
1804   S  L  N  Y  -
```

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B

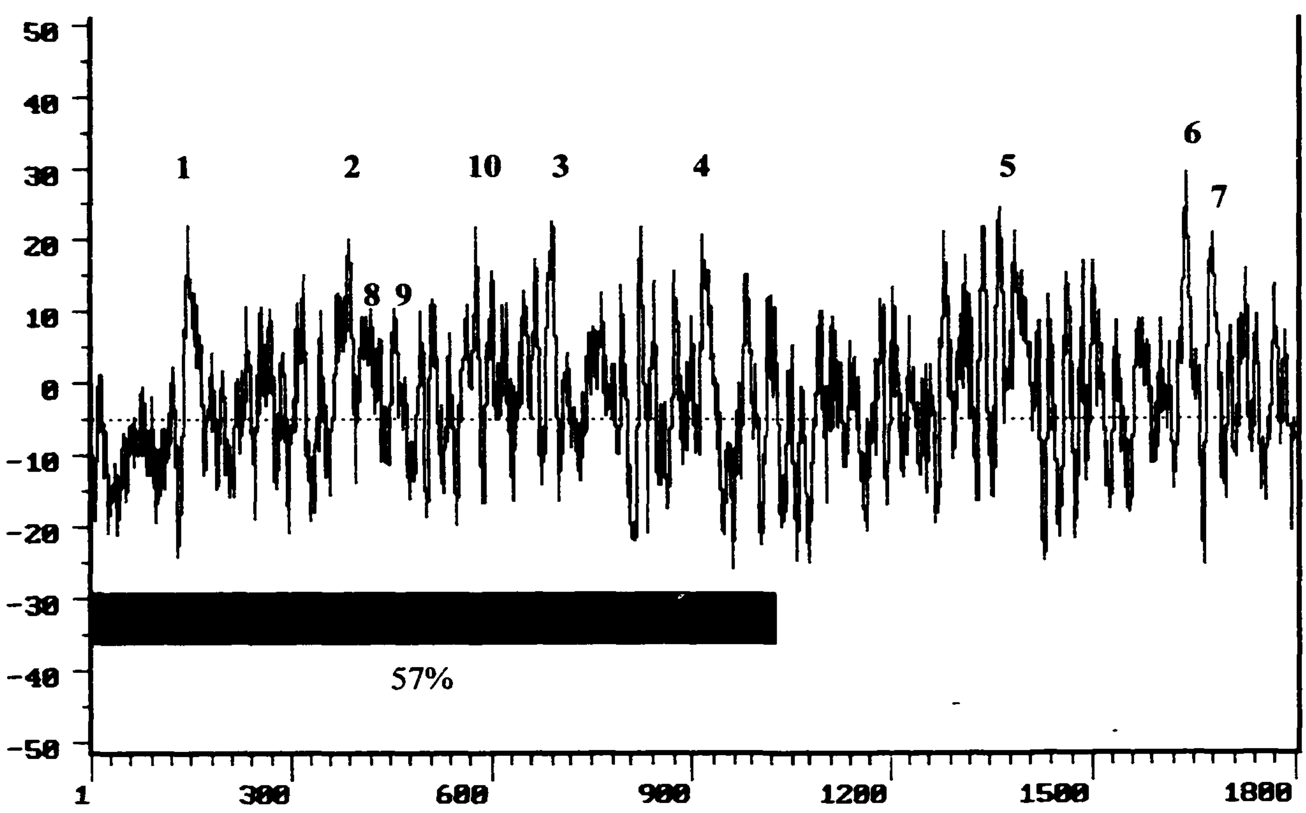

Figure 2-3. ***BLM3*** **nucleotide sequence, the predicted amino acid sequence and hydropathy plot. (A)** ***BLM3*** **nucleotide sequence and the predicted amino acid sequence. The region complementing the** ***blm3-1*** **mutation is denoted between the two * symbols. The amino acid sequence is shown in the one-letter code and residues are numbered at the left; nucleotide positions are numbered at right. The predicted transmembrane domains are underlined. (B) Numbers 1-7 denote the transmembrane domains predicted by PC gene and 1-10 denote the transmembrane domains denoted by Entrez. ■ 57% of the cloned insert from pPM311 which complemented the** ***blm3-1*** **mutation.**

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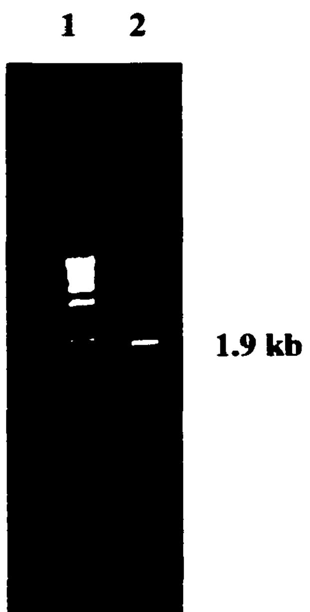

Figure 3-1. PCR verification of the cassette containing GFP (S65T mutant version of GFP) and the selectable marker *HIS3*. The GFP-HIS3 cassette cloned into pBM3412 was amplified by PCR using forward and reverse primers (Table 3). Lane 1: λ Hind, Lane 2: 1.9 kb GFP-*HIS3* cassette used for transformation.

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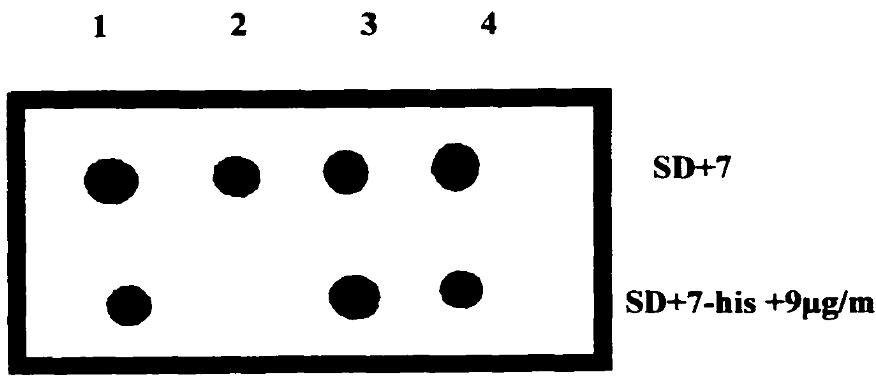

Figure 3-2. Blm3p-GFP phleomycin resistant transformants. The Blm3p-GFP transformants (CM1522-9A containing *blm3::gfp-HIS3*) were grown and tested for resistance on SD+7 and SD+7- his + 9 μg/ml of phleomycin. Both transformants were resistant to phleomycin. Lane 1: *BLM3:* (wild-type control), Lane 2: *blm3-1:* (mutant control), Lanes 3-4: Blm3p-GFP transformants.

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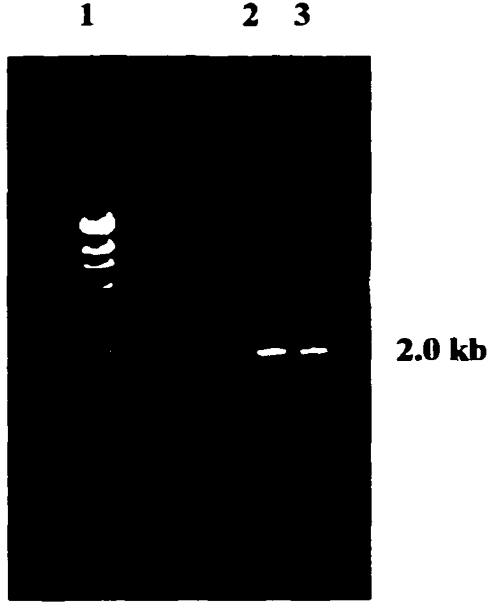

Figure 3-3. Verification of the correct GFP integration fused to the 3'end of *BLM3*. Two test primers (Table 3) were used to amplify genomic DNA from two GFP-HIS3 transformants (CM1522-9A containing *blm3::gfp-HIS3*) which yielded the correctly integrated transformants. Lane 1: λHind, Lanes 2-3: Blm3p-GFP transformants with correct GFP integration fused to the 3'end of *BLM3*.

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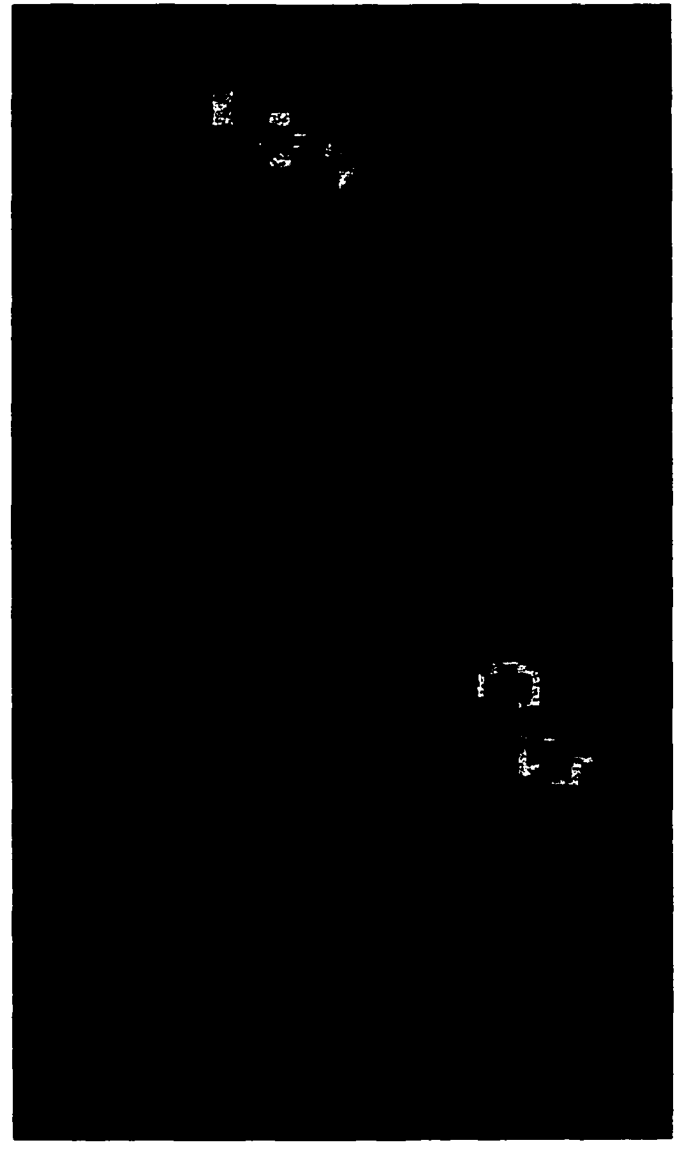

Figure 3-4. Confocal micrograph of Blm3p-GFP. Living yeast cells expressing GFP viewed by confocal microscopy of Blm3p-GFP. Yeast cells were grown in liquid YPAD medium overnight at 30°C and incubated with 5 μg/ml of phleomycin for 1 hour.

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Figure 3-5. PCR verification of the *HIS3* disruption cassette in the pRS303 plasmid. lane 1: λ Hind; lane 2: (+) positive control; lane 3: negative control; lanes 4-8: 1.1 kb *HIS3* disruption cassette in the pRS303 plasmid.

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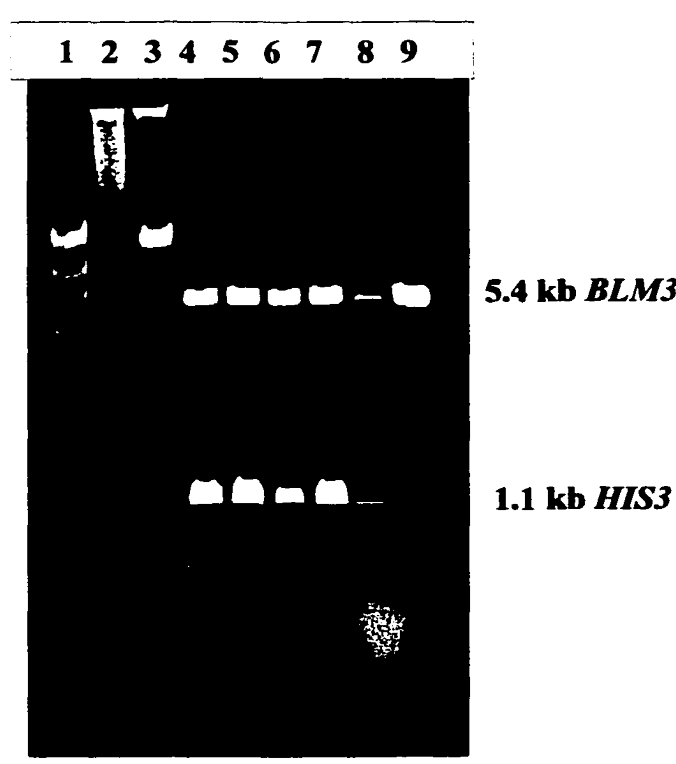

Figure 3-6. Analytical PCR verification for the disruption of *BLM3*. The test primers (Table 2) give a 5.4 kb (*BLM3*) and 1.1 kb (*HIS3*) DNA fragment size, confirming the disruption took place. Lane 1 H: λ_H; Lanes 2 and 3 (+): positive control, lanes 4-8 (CM-1521, CM-1522, CM-1523, CM-1524, CM-1525): diploid strains bearing the null allele of *BLM3* in the heterozygous configuration; lane 9 BMA.

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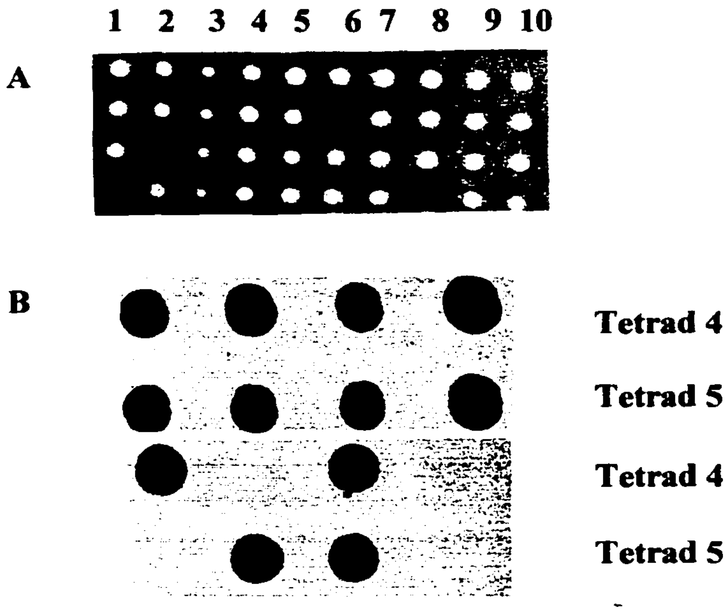

Figure 3-7. Tetrad analysis of the *BMA1* diploid strain transformed with the disruption cassette corresponding to *BLM3*. Tetrad analysis from one of the sporulated *HIS3*$^{+}$ prototrophic diploids (CM-1522) is illustrated. A) Dissection plate of 10 asci on YPAD showing the viability of *BLM3* and *blm3Δ*, indicating nonessentiality of *BLM3*. B) Haploid strains derived from 2 complete tetrads (tetrads 4 and 5) were analyzed for growth on SD+7 (top) and SD+7-his (bottom) plates. Mendelian segregation of the *HIS3*+ phenotype was observed in all.

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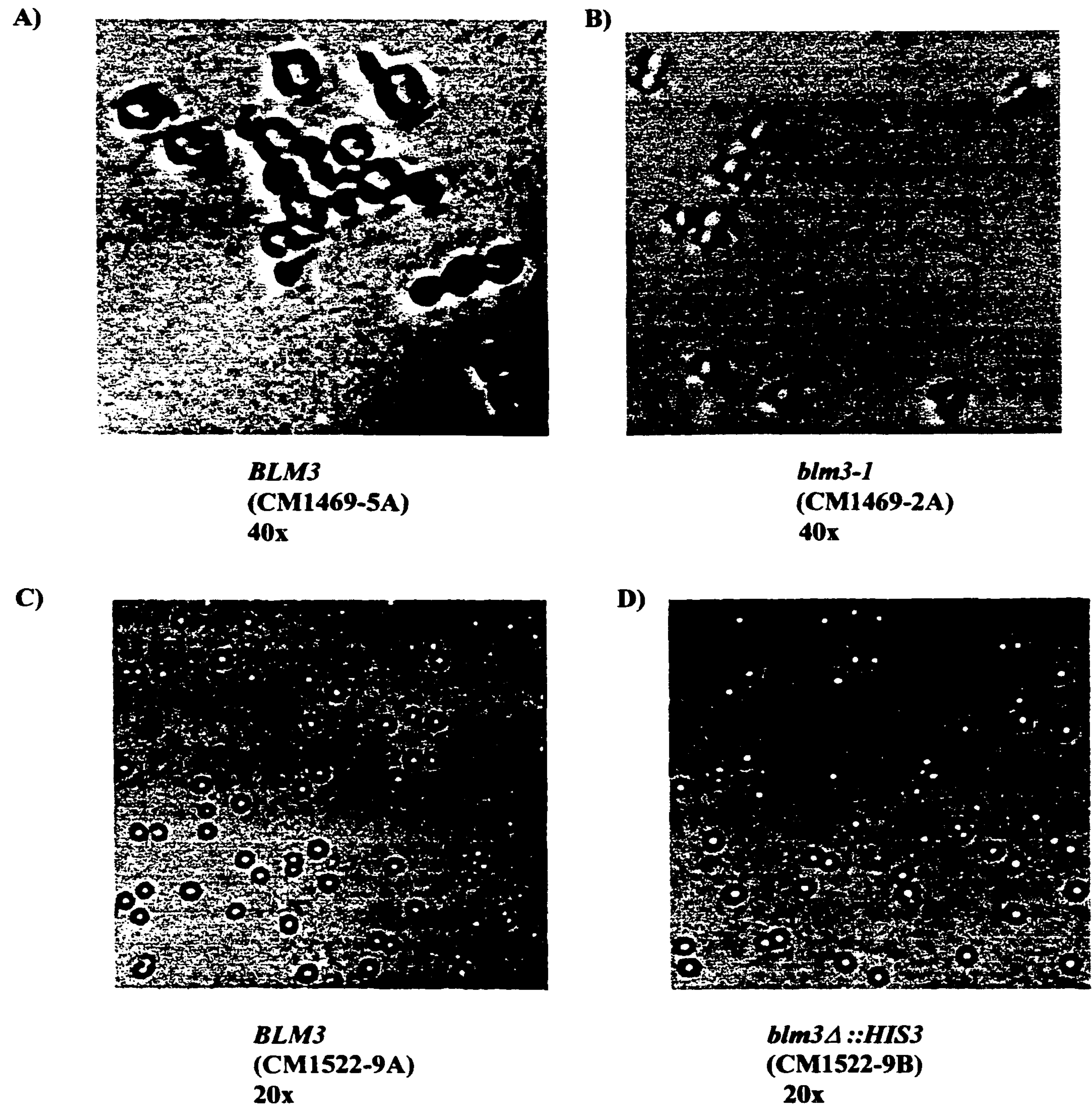

Figure 3-8. Light micrographs comparing cell sizes of A) *BLM3* (CM1469-5A) and B) *blm3-1* (CM1469-2A), 40x magnification. *BLM3* cells are larger than *blm3-1* cells. C) *BLM3* (CM1522-9A) and D) *blm3-Δ::HIS3* (CM1522-9B), 20X magnification. There were no significant differences in the sizes of *blm3-Δ::HIS3* and *BLM3* cells.

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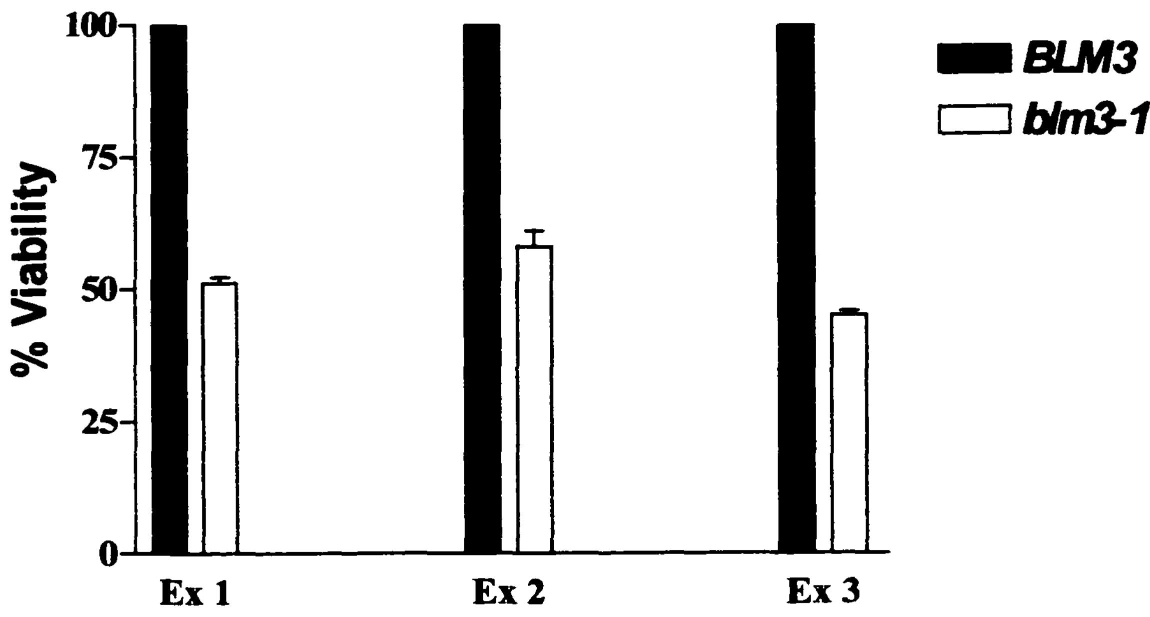

Figure 3-9. Reduced viability for *blm3-1* cells compared to *BLM3*. In three separate experiments, *blm3-1* (CM1469-5C) cells displayed a decrease in viability compared to *BLM3* (CM1469-5A) cells. Cells were grown from 10^4 cells/ml to 6 x 10^7 cells/ml, suspended in deionized water, plated on YPAD (100 cells/plate on 5 separate plates).

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A)

■ *BLM3*
▲ *blm3-1*

CELLS/ML

10^9 10^8 10^7 10^6 10^5 10^4 10^3 10^2 10^1 10^0

0 10 20 30 40 50 60 70 80

TIME (hours)

B)

■ *BLM3*
▲ *blm3-1*

CELLS/ML

10^9 10^8 10^7 10^6 10^5 10^4 10^3 10^2 10^1 10^0

0 10 20 30 40 50 60 70 80

TIME (hours)

Figure 3-10. Comparisons of growth curves A: *BLM3* (CM1469-5A) and *blm3-1* (CM1469-5C) strains. B: *BLM3* (CM1522-9A) and *blm3*Δ (CM1522-9B) strains. Growth of strains in liquid media (YPAD) was determined by hemacytometer counts.

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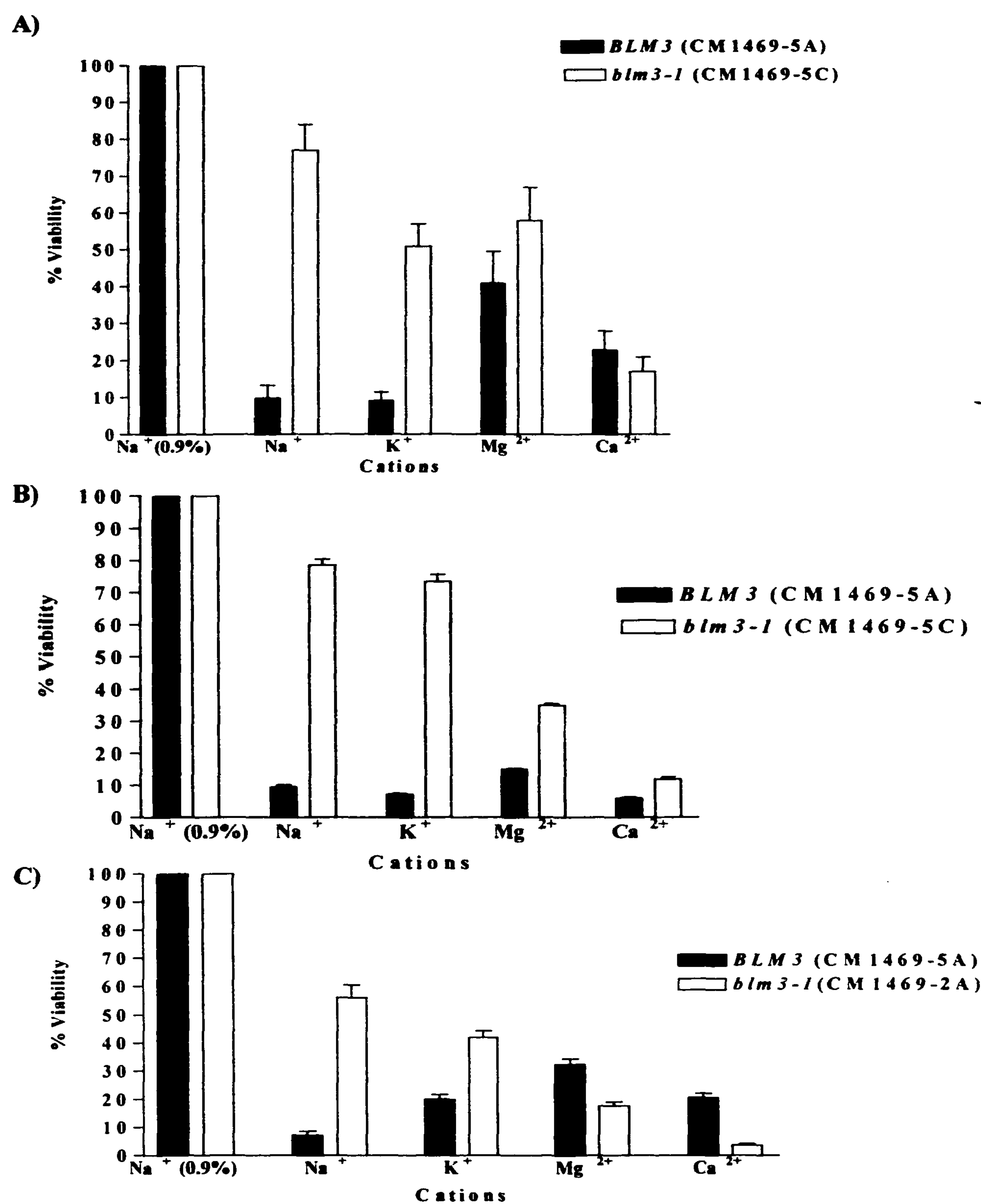

Figure 3-11. Strain viability of *BLM3* and *blm3-1* strains after exposures to cationic solutions (NaCl, KCl, $MgCl_2$, $CaCl_2$). In three separate experiments (A, B, and C) the *blm3-1* mutant exhibited higher survival than *BLM3* cells after exposures to 2 M Na^+ and K^+. All values are relative to 0.1 M saline control.

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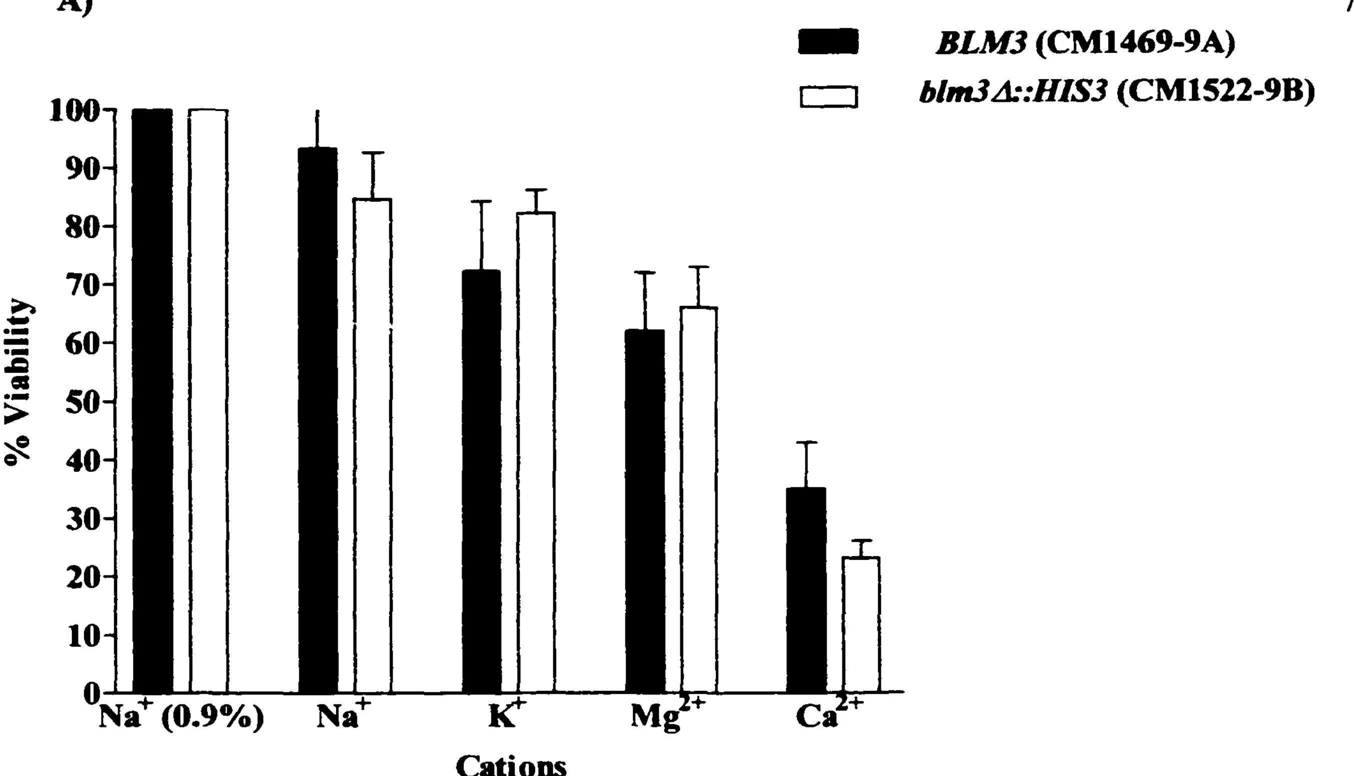

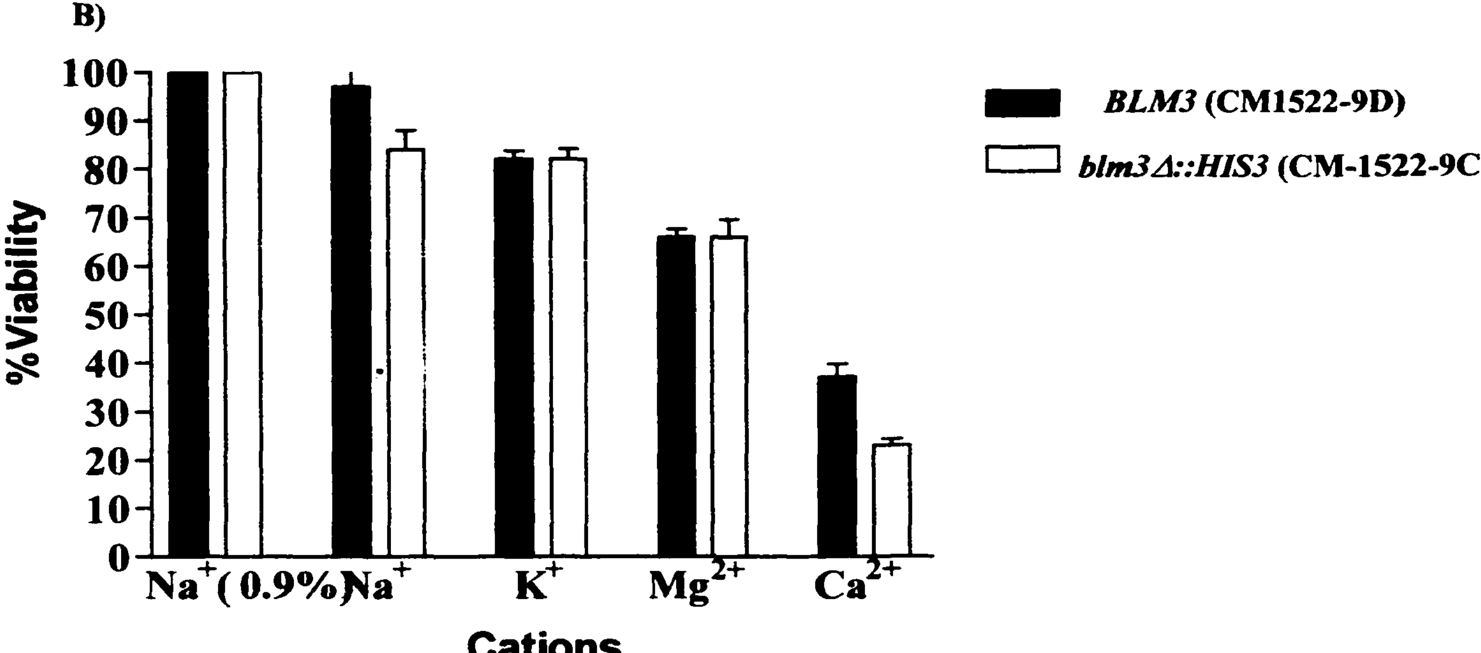

Figure 3-12. Strain viability of *BLM3* and *blm3Δ::HIS3* strains after exposure to cationic solutions (NaCl, KCl, $MgCl_2$, $CaCl_2$). In two separate experiments (A, B), the *blm3Δ::HIS3* mutant and related *BLM3* strains were not significantly different. All values are relative to 0.1 M saline control.

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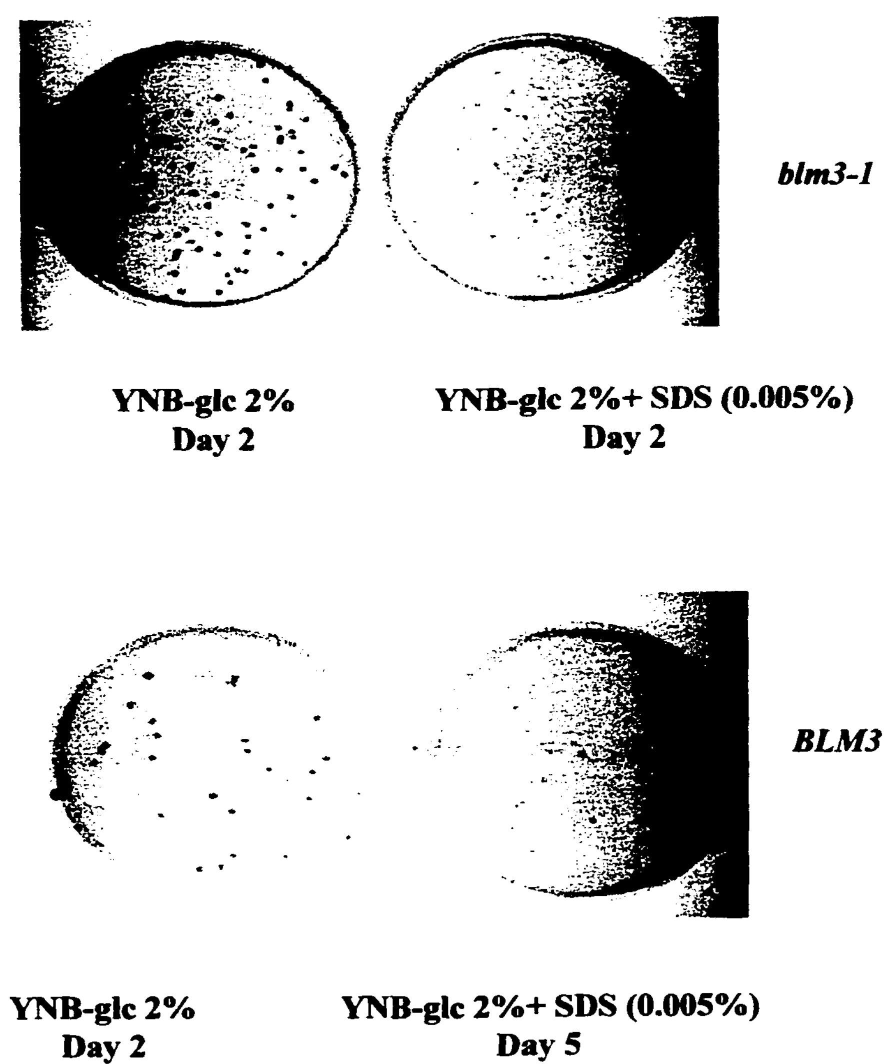

Figure 3-13. Growth of *BLM3* and *blm3-1* cells with and without SDS (0.005%). Mutant *blm3-1* and *BLM3* strains were grown on YNB-glc 2% and YNB-glc 2%+ SDS (0.005%). *BLM3* and *blm3-1* cells both grew on day 2 on YNB-glc 2%. Mutant *blm3-1* cells grew on YNB-glc 2%+ SDS (0.005%) with slightly smaller colonies compared to *blm3-1* cells grown on YNB-glc 2% on day 2. In contrast, *BLM3* colonies were not observed until day 5. *BLM3* cells recovered on day 5 compared to *blm3-1* cells that recovered on day 2 on YNB-glc 2%+ SDS (0.005%).

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