

Meiotic and Mitotic Phenotypes Conferred by the *blm1-1* Mutation in *Saccharomyces cerevisiae* and *MSH4* Suppression of the Bleomycin Hypersusceptibility

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Abstract: Oxidative damage can lead to a number of diseases, and can be fatal. The *blm1-1* mutation of *Saccharomyces cerevisiae* confers hypersusceptibility to lethal effects of the oxidative, anticancer and antifungal agent, bleomycin. For the current report, additional defects conferred by the mutation in meiosis and mitosis were investigated. The viability of spores produced during meiosis by homozygous normal *BLM1/BLM1*, heterozygous *BLM1/blm1-1*, and homozygous mutant *blm1-1/blm1-1* diploid strains was studied and compared. Approximately 88% of the tetrads derived from homozygous *blm1-1/blm1-1* mutant diploid cells only produced one or two viable spores. In contrast, just one tetrad among all *BLM1/BLM1* and *BLM1/blm1-1* tetrads only produced one or two viable spores. Rather, 94% of *BLM1/BLM1* tetrads and 100% of *BLM1/blm1-1* tetrads produced asci with four or three viable spores. Thus, at least one copy of the *BLM1* gene is essential for the production of four viable spores after meiosis. During mitotic growth, mutant *blm1-1* strains grew at reduced rates and produced cells with high frequencies of unusual morphologies compared to wild-type strains. These results indicated *BLM1* is also essential for normal mitotic growth. We also investigated the suppression by the *MSH4* gene, a meiosis-specific MutS homolog, of the bleomycin hypersusceptibility of *blm1-1* mutant cells, and the relationship of *MSH4* to *BLM1*. We screened a genomic library, and isolated the *MSH4* gene on the basis of its ability to suppress lethal effects of bleomycin in *blm1-1* cells. However, genetic mapping studies indicated that *BLM1* and *MSH4* are not the same gene. The possibility that chromosomal nondisjunction could be the basis for the inability of *blm1-1/blm1-1* mutant cells to produce four viable spores after meiosis is discussed.

Keywords: *Saccharomyces cerevisiae*, bleomycin, *blm1-1*, meiosis, *MSH4*.

Introduction

The structures of bleomycins and structurally-related phleomycins are shown in Figure 1. These low molecular weight compounds cause single- and double-stranded breaks in DNA [1] and damage to fungal cell walls [2]. They bind to DNA and generate free radicals in an oxygen-dependent, metal-dependent reaction [3-5]. Bleomycins and phleomycins are radiomimetic in that they produce effects similar to those observed after exposure of DNA to ionizing radiation [6-9]. As a result, the drugs are employed as tools in studies of oxidative damage to cells [10]. To gain knowledge about how cells respond to oxidative damage, we isolated a series of mutants in *Saccharomyces cerevisiae* that confer hypersensitivity to killing by the bleomycins and phleomycins. One of these mutants contained the recessive *blm1-1* mutation [11,12].

Additional defects conferred by *blm1-1* were investigated for the current report. We also describe the cloning and isolation of the *MSH4* gene, essential for wild-type spore viability in *S. cerevisiae* [13]. The Msh4 protein is a homolog of the MutS DNA mismatch repair protein in bacteria [14]. Tetrad analyses indicated that the *BLMI* gene is essential for the production of four viable spores after meiosis, and mitotic analyses indicated *BLMI* is essential for normal mitotic growth.

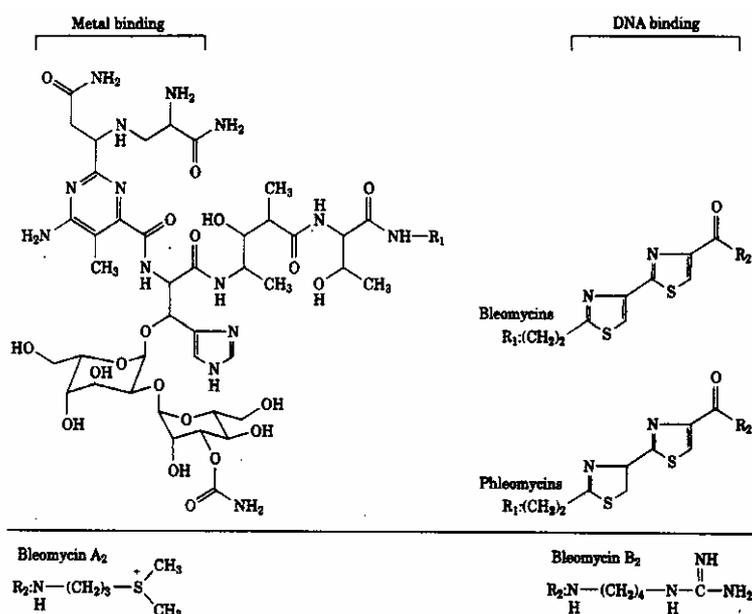


Figure 1. Structures of bleomycins and phleomycins. Figure taken from Moore, 1999.

Materials and Methods

Strains and Plasmids

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

Table 1. Strains and plasmids used.

Strain or Plasmid	Genotype or Selectable Markers	Source
<i>Saccharomyces cerevisiae</i>		
CM1457-79A	<i>MATa ade2-1 and/or ade2-40 trp1-1 and/or trp5b</i>	This laboratory
CM1457-79B	<i>MATa ade2-1 and/or ade2-40 ilv1-92 trp1-1 and/or trp5b</i>	This laboratory
CM1457-79C	<i>MATa ade2-1 and/or ade2-40 blm1-1 trp1-1 and/or trp5b leu2-3 and/or leu2-112 ura3-1</i>	This laboratory
CM1457-79D	<i>MATa ade2-1 and/or ade2-40 blm1-1 ilv1-92 leu2-3 and/or leu2-112 trp1-1 and/or trp5b ura3-1</i>	This laboratory
PS593/6H	<i>MATa his3Δ200 leu2-3 or leu2-112 msh4::LEU2 trp1-289 ura3-52</i>	Dr. Nancy Hollingsworth; Dr. Shirleen Roeder [15]
Plasmids		
YCp50	amp tet ARS1 CEN4 URA3	Johnston and Davis, 1984 [16]
pPM118	tet ARS1 CEN4 URA3	This laboratory
pPM118-4	tet ARS1 CEN4 URA3	This laboratory

Media and Growth Conditions

Non-synthetic complete solid medium (YPAD), containing 2% glucose, 2% Bacto-peptone, (Difco Laboratories, Detroit, Michigan), 1% Bacto-yeast extract (Difco), 2% Bacto-agar (Difco), and 0.16 mg/ml adenine sulfate were used for the nonselective growth of all yeast strains. If liquid YPAD medium was required, agar was not included in the preparation. Solid synthetic medium (SD) was used for the selective growth of desired strains. It contained 0.2% Bacto-yeast nitrogen base (Difco), 0.5% ammonium sulfate, 2% glucose, and 2.5% Bacto-agar. For strain selection or for the growth of individual strains during screening procedures, the specific amino acid requirements of each strain were added during the preparation of SD medium.

Sporulation and Dissection of Tetrads

The steps involved in mating of haploid strains to produce diploid cells, sporulation of diploids, and analysis of tetrads after meiosis are outlined in Figure 2. The yeast haploids of opposite mating types were crossed, and the resulting diploids were selected on supplemented SD media. A colony from the selected diploids was inoculated into sterile liquid YPAD medium and incubated with aeration at 30°C overnight. Cells were grown to stationary phase, then inoculated at a cell density of 1000 cells/ml into presporulation media containing 0.8% Bacto-yeast extract (Difco), 0.3% Bacto-peptone (Difco), 10%

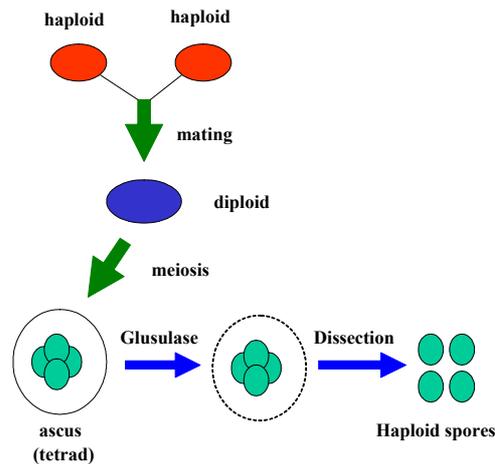


Figure 2. Flow chart outlining the steps involved in meiosis and the production of ascospores in *S. cerevisiae*. Haploid strains of opposite mating types were mated to produce diploid cells. Diploid cells were then sporulated, and ascus cell walls were treated with the lytic enzyme, glusulase. The treated asci or tetrads were microdissected to release and analyze the four spore products of meiosis.

glucose, and 0.16 mg/ml adenine sulfate. The culture was incubated at 23°C until the cells grew to a density of 1×10^7 to 5×10^7 cells/ml (mid-exponential growth). Cells were pelleted, washed twice, and resuspended into sporulation media containing 1% potassium acetate and the amino acids required for sporulation of the diploids. The tetrads obtained after sporulation were dissected using a micromanipulator (Singer Instruments Co. Ltd., Somerset, England) and incubated on solid YPAD at 30°C.

Zeocin Treatments

Zeocin is a 20-fold dilution of phleomycin, and was purchased from Invitrogen (Carlsbad, CA). Aliquots from the stock solution of Zeocin were added to the desired concentration in SD or YPAD media which had been sterilized and cooled to approximately 45°C. Zeocin medium was always prepared within 24 hours prior to use. Yeast strains listed in Table 1 were replica-plated on Zeocin media and grown for 3 days at 30°C to determine their susceptibility to killing by the drug.

E. coli and Yeast Transformations

E. coli transformation was carried out according to the CaCl_2 method described by Sambrook *et al.* [17]. Transformation of yeast cells was carried out using lithium acetate in the method of Ito and co-workers [18] and as modified by Rose [19] and Rose and Broach [20].

Results

Reduced Viability of Spores from Homozygous Mutant Strains

The viability of spores from homozygous *blm1-1/blm1-1* mutant strains was compared to that of spores from *BLM1/BLM1* homozygous normal strains and heterozygous *BLM1/blm1-1* strains. The homozygous normal diploid was created by crossing two wild-type haploid segregants, CM1457-79A and CM1457-79B (Table 1). The heterozygous diploid was created by crossing a wild-type haploid, CM1457-79A, to a mutant haploid, CM1457-79C. The homozygous mutant diploid was created by crossing two mutant haploids, CM1457-79C and CM1457-79D. Each diploid was sporulated, and the tetrads obtained from each sporulated diploid were dissected (Figure 2).

The viability of the spores in each tetrad is tabulated in Table 2. Ninety-four percent of the tetrads from the *BLM1/BLM1* diploid and 100% of the tetrads from the *BLM1/blm1-1* diploids produced four or three viable spores. In contrast, no tetrads from *blm1-1/blm1-1* cells produced asci with four viable spores, and only 13% of the tetrads produced three viable spores. One- or two-spored viability was predominant. Thus, in the presence of one or two copies of the *BLM1* gene (as in homozygous or heterozygous diploids), tetrads appear capable of completing meiosis and producing four viable spores. However, in the absence of the *BLM1* gene, cells appear unable to complete meiosis and produce four viable spores. These results indicate that the *BLM1* gene is essential for the viability of all four spores after meiosis, and that the *blm1-1* mutation confers a deficiency in meiosis since the homozygous mutant cells are unable to produce four viable spores.

Table 2a. Viability of spores from *BLM1/BLM1*, *BLM1/blm1-1*, and *blm1-1/blm1-1* diploid strains

Diploid Type	Total number of tetrads dissected	No viable spores	1 viable spore	2 viable spores	3 viable spores	4 viable spores
Homozygous normal	16	0	1	0	6	9
Heterozygous	14	0	0	0	5	9
Homozygous mutant	31	0	7	20	4	0

Table 2b. Percentage of viable spores produced by each genotype

Diploid Type	Total number of tetrads dissected	Sporulation (%)	1 viable spore (%)	2 viable spores (%)	3 viable spores (%)	4 viable spores (%)
Homozygous normal	16	70±5.8 ¹	6	0	38	56
Heterozygous	14	66.3±4.5 ¹	0	0	36	64
Homozygous mutant	31	52.1±4.1 ¹	23	65	13	0

¹Standard errors of the means were calculated from three to five replicates.

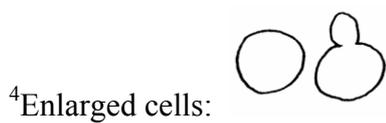
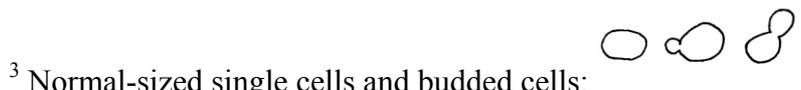
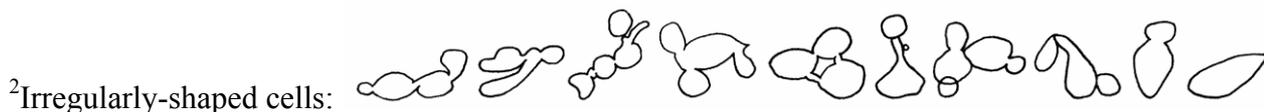
Mitotic Phenotype of blm1-1 Mutant Strains: Reduced Growth Rate and Elevated Frequencies of Irregularly-shaped Cells

Mutant *blm1-1* strains were found to grow at reduced rates compared to the wild-type strains. At stationary phase, the titers of *blm1-1* haploid strains were approximately 25% less than those of *BLM1* cells (Table 3). During growth, the *blm1-1* mutant cells also assumed peculiar shapes at high frequencies. When quantitated, approximately five percent of *blm1-1* cells in stationary-phase populations were abnormally shaped. In addition, twice as many cells appeared swollen in *blm1-1* populations than in *BLM1* populations.

Table 3. Comparisons of Growth and Abnormally-shaped Cells in *BLM1* and *blm1-1* Strains ¹

Strains	Final Titters (cells/ml)	Number and Percent of Irregularly-shaped Cells ²	Normal-sized Single Cells and Budded Cells ³	Number and Percent of Enlarged Cells ⁴	Total Number of Cells
Means of experiments one and two					
<i>BLM1</i> (CM1457-79A)	5.00 x 10 ⁸	0%	(462) 84.6%	(84) 15.4%	546 (100%)
Experiments three and four: means of both strains					
<i>blm1-1</i> (CM1457-79C and CM1457-79D)	3.60 x 10 ⁸	(28.5) 5.23%	(350) 64.1%	(167) 30.6%	546 (100%)
Experiments five and six: means of both strains					
<i>blm1-1</i> (CM1457-79C and CM1457-79D)	3.78 x 10 ⁸	(29) 5.31%	(347) 63.6%	(170) 31.1%	546 (100%)

¹Each cell was classified based on the following criteria and relative sizes.



Isolation of Plasmids, Restriction Analyses, and DNA Sequence Analyses

In addition to the meiotic defect, the *blm1-1* mutation confers hypersensitivity to lethal effects of bleomycin [21]. Thus, this phenotype was used to isolate plasmids that relieved the drug hypersensitivity. Isolation of the pPM118 and pPM118-4 plasmids from a *S. cerevisiae* (S288C) genomic library was previously described [22]. A ³²P-labelled 3.8 kb *Bam*HI-*Cla*I fragment from pPM118 was used as a probe to screen the genomic library and isolate pPM118-4.

To identify the complete sequences of the inserts in pPM118 and pPM118-4, approximately 200 bases at each end of the inserts were sequenced by two custom-made primers originating from the vector sequences. These four end sequences were then used to search the nucleotide sequence databases through the National Center for Biological Information (NCBI) using the BLAST program [23]. These sequences were also used to search the SGD (*Saccharomyces* Genomic Database). The search results indicated the cloned sequence on pPM118 contains a region from chromosome VI between 133748 and 140147 bases, including the full *MSH4* gene (Figure 3). Restriction maps, chromosomal origin and the overlapping region of pPM118 and pPM118-4 are shown in Figure 3, along with the identification and location of the open reading frame of the *MSH4* gene.

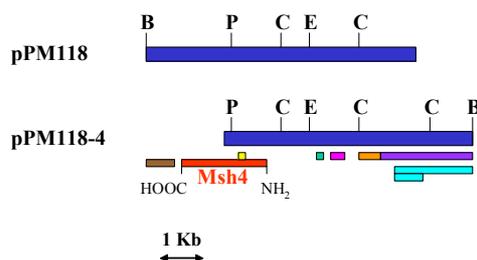


Figure 3. Restriction maps of the inserts of the plasmids pPM118 and pPM118-4, and the location of the *MSH4* gene. Restriction maps of the two chromosomal inserts are shown with their corresponding overlapping region. B: *Bam*HI, C: *Cla*I, E: *Eco*RI, P: *Pst* I. Inserts originated from chromosome VI and the coordinates for the entire region cloned on pPM118 and pPM118-4 extends from 133748 to 141542 bases. Map of chromosomal features from SGD: VTC2 (brown), ARS605 (yellow), Msh4 (red), tRNA-Asn (green), Ty1LTR (pink), Ty2LTR (orange), Ty2 (purple), TyAGag (aqua), TyB (aqua).

Relief of Drug Hypersensitivity

The relief of the hypersensitivity to killing by bleomycin conferred by the pPM118-4 plasmid is illustrated in Figure 4. For comparison, the figure also illustrates the resistance conferred by the wild-type or normal *BLM1* gene and the hypersensitivity conferred by the mutant *blm1-1* gene (mutant *blm1-1* strains transformed with the YCp50 vector alone). The drug resistance conferred by pPM118-4 is comparable to the resistance of wild-type cells at all drug concentrations with the exception of the

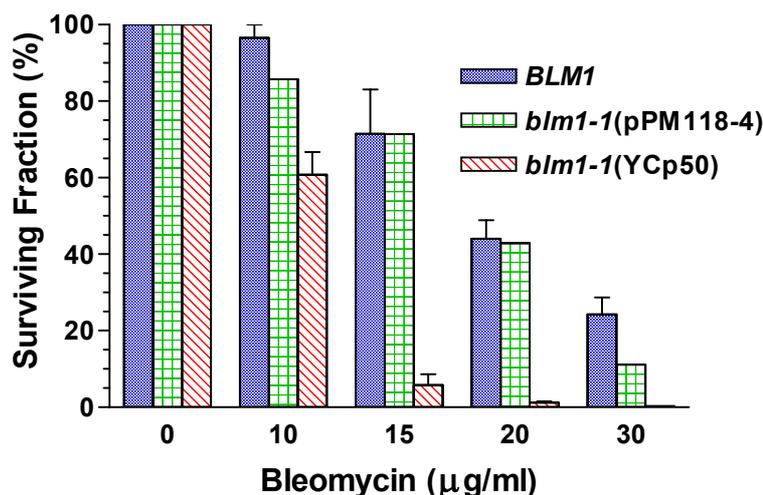


Figure 4. A representative comparison of the resistance of normal (*BLM1*, CM1401-5B) and transformed *blm1-1* mutant strains to lethal effects of bleomycin. The *blm1-1* mutant strain, CM1401-5C, was transformed with the plasmid (pPM118-4) or with the YCp50 vector alone. The normal segregant and the transformed mutant strains were plated on supplemented SD medium containing various concentrations of bleomycin. Plates were incubated at 30°C for 3 days. The survival value of the last cell is $0.3 \pm 0.2\%$.

very high concentration of 30 µg/ml bleomycin. The resistance provided by pPM118-4 is also high at 30 µg/ml bleomycin, though somewhat less than wild-type cells. There are several possible reasons for the somewhat decreased resistance relative to wild-type cells at the highest drug dose. For example, if *MSH4* is a suppressor of the *blm1-1* phenotype, rather than the same gene as *BLM1*, it may not be able to fully relieve the drug hypersusceptibility of mutant cells at high drug concentrations. Second, whether *MSH4* and *BLM1* are or are not the same gene, the full gene may be required on the plasmid to obtain full complementation. Third, the encoded protein may not be produced as efficiently from the plasmid as it is from the chromosome. Fourth, the region of the gene on pPM118 may contain an alteration.

Genetic Mapping Showed that MSH4 is not the BLM1 Structural Gene

The *MHS4* gene has one function that is similar to that of the *BLM1* gene — both genes are required in meiosis for wild-type levels of spore viability in *S. cerevisiae*. Thus, to determine if *BLM1* and *MSH4* are the same gene, linkage analysis was carried out by genetic mapping to determine if *MSH4* is linked to (and thus could be) the *BLM1* gene.

A mutant strain, PS593/6H, with the *LEU2* marker replacing nucleotides 168-2290 of the *MSH4* coding region [15], was kindly provided by Dr. Nancy Hollingsworth (Department of Biochemistry and Cell Biology, State University of New York at Stony Brook, Stony Brook, NY). This strain was

crossed to a *leu2/blm1-1* strain, CM1457-79C. The resulting diploid was genotypically *MSH4/msh4::LEU2, BLM1/blm1-1*. The *LEU2* gene was used to track the *msh4* gene in linkage analysis, and Zeocin hypersensitivity was used to track the *blm1-1* mutation. If the two genes are the same, the genotypes in the tetrad segregants would be one or the other of the parental ditypes (PD). That is, there will be 2:2 segregation of *LEU2* (leucine prototrophy) with *BLM1* (wild-type or normal drug resistance), and *leu2* (leucine auxotrophy) with the *blm1-1* mutation (drug hypersensitivity).

Segregation of *BLM1*, *blm1-1*, *MSH4* and *msh4::LEU2* in meiosis is shown in Table 4. The segregation pattern from the tetrad analysis of the cross PS593/6H x CM1457-79C revealed that 35.7% of the tetrads were PD, 42.9% of the tetrads were non-parental ditypes (NPD; the genotypes of all four spores are different from the genotypes of the parental haploids), and 21.4% were tetratypes (TT; two spores are parental genotypes and two spores are not). For two genes to be linked, the ratio of PD:NPD: TT is >1: <1:0 [24]. The ratio obtained from this assay is <1:1<1. Actually, the ratio of each genotype among the total spores is close to 1:1:1:1, consistent with random assortment of each gene on its respective chromosome. Thus, the linkage analysis indicates that *MSH4* and *BLM1* are different genes.

A master plate containing solid YPAD medium and inoculated with parental strains, all the spores from dissected tetrads, and positive controls were created. Cells from this plate were replica-plated on SD+7 media lacking leucine and on complete media containing Zeocin. The *MSH4* gene was tracked by the wild-type LEU+ phenotype from PS593/6H, and the *BLM1* phenotype was introduced into the cross by PS593/6H.

Table 4. Analysis of tetrad segregation of CM1457-79C (*blm1-1,leu2*) x PS593/6H (*msh4::LEU2, BLM1*). (The number of spores of each genotype is tabulated. Only results from four-spored asci are tabulated)

Classes of tetrads	Number of tetrads	Number of spores	<i>blm1-1, leu2</i>	<i>BLM1, LEU2</i>	<i>BLM1, leu2</i>	<i>blm1-1, LEU2</i>
Parental Ditype (PD)	5	20	10	10	0	0
Non-Parental Ditype (NPD)	6	24	0	0	12	12
Tetratype (TT)	3	12	3	3	3	3
Total spores	14	56	13	13	15	15

Discussion

Analyses of the meiotic and mitotic phenotypes conferred by the *blm1-1* mutation indicate that in addition to the bleomycin and phleomycin hypersusceptibility, the *blm1-1* mutation confers defects in meiosis and mitosis. These results have not previously been reported. It is not known at the present time what mechanism accounts for the low spore viability after meiosis in homozygous *blm1-1/blm1-1* mutant diploid cells. Compared to the homozygous normal *BLM1/BLM1* and heterozygous *BLM1/blm1-1* diploid strains, sporulation was not dramatically lower in the homozygous mutant cells (Table 2). But the fact that only one or two spores from 88% of the tetrads survived indicates some step in meiosis is likely to be defective. One possibility for the defect is that nondisjunction of one or more chromosomes occurs at high frequency during meiosis in the mutant diploids. A failure of chromosomes to properly separate could occur at either the first or second meiotic division, as shown in Figure 5. If the nondisjunction were limited to a specific chromosome, some spores would receive an extra chromosome and some spores would not receive that chromosome (Figure 5). If the nondisjunction were not limited to a single chromosomal pair, different homologous chromosomes would fail to properly separate during meiosis. Either situation could lead to defective meiotic products and an inability to produce four viable spores. Nondisjunction events during mitosis might account for the *blm1-1/blm1-1* mitotic phenotypes of reduced growth rates and high frequencies of abnormal cell morphologies. The decreased growth rates may actually reflect the inviability of some of the cells during mitotic growth.

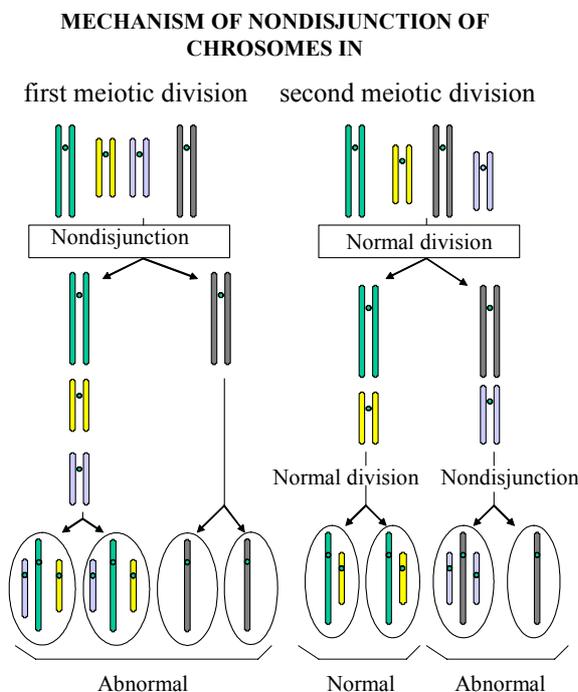


Figure 5. An illustration of the fates of two chromosomal pairs during meiosis. One pair of homologous chromosome behaves normally as it goes through meiosis I and meiosis II. The other pair does not disjoin in either the first or the second meiotic division.

Disturbance of the structural components of the synaptonemal complex in meiosis can lead to nondisjunction of homologous chromosomes [25]. Although the relationship between the Blm1p and nondisjunction is unknown, it is known that Msh4p functions in the formation of the synaptonemal complex [15]. The *MSH4* gene in *S. cerevisiae* encodes a protein involved in crossing over between chromosomes in meiosis, and the gene is not transcribed in mitosis [25]. Recently, Msh4p was shown to function in chromosomal synapsis in meiosis, and regulate the distribution of meiotic crossovers along chromosomes [15]. The protein localizes to discrete sites in meiotic chromosomes when synapsis initiates, and promotes formation of the synaptonemal complex [15].

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