The Functions of the MSH2 and MLH1 Proteins during Meiosis in

Tetrahymena thermophila

by

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M.Sc., Delft University of Technology, Leiden University, the Netherlands, 2005

B.Sc., Wuhan University, Wuhan, China, 2003

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ABSTRACT

Msh2 and Mlh1 proteins from *Tetrahymena thermophla* are homologues of MutS and MutL from *Escherichia coli* respectively. MutS and MutL are DNA mismatch repair proteins. In eukaryotes, MutS homologues recognize the replication errors and MutL homologues interact with MutS homologues and other proteins to make the repair occur. Biolistic transformation has been done to make the msh2 and mlh1 single knockouts in

the macronuclei of different strains and the knockouts were verified complete. Two strains of WT crossing KO or KO crossing KO, with different mating types, were induced to conjugate. The processes were studied by microscopy using DAPI staining. For the *msh2* knockouts, there were no crescent micronuclei formed throughout the conjugation of two knockout cells, and the pairing level was reduced severely. However, a knockout cell and a wild-type cell could conjugate normally at a high level pairing efficiency. Msh2 protein seems to be important to cell pairing and indispensible for the formation of the crescent micronuclei during cell conjugation. For the *mlh1* knockouts, the pairing level of a knockout and a wild-type was reduced by half and the pairing level of two knockouts was reduced more than 80%; however, the paired cells in both could complete the conjugation with delay. Pms2 protein may have redundant roles in the MutL heterodimer (Mlh1-Pms2). In addition, chemical mutagens treated knockout was crossed with non-treated wild-type and the conjugation was compared with treated wild-types. Most of the treated knockout cells could not pair after starvation and mixing with non-treated wild-type cells, which means most of the cells could not enter meiotic phase. It is probable that G2/M checkpoint arrested the meiotic cell cycle and the intra-S phase was inactivated. Thus, Msh2 protein may have a role in the meiotic intra-S phase checkpoint system.

TABLE OF CONTENTS

PRELIMINARY PAGES

Supervisory Committee……...……ii

Abstract……...iii

Table of Contents…….v

List of Tables……..ix

List of Figures……..x

List of Abbreviations…….xii

Acknowledgements…..……..…….……..xiii

1 INTRODUCTION……...1

1.1 DNA Mismatch Repair System……...4

1.1.1 Mechanism of mismatch repair ……..……..…5

1.1.2 Eukaryotic mismatch repair enzymes……...7

1.2 *Tetrahymena thermophila* Model……...……..……...……9

1.2.1 Macronucleus (MAC) of *T. thermophila*……...….10

1.2.2 Micronucleus (MIC) of *T. thermophila* …….12

1.3 *Tetrahymena thermophila* Meiosis……...……...…..13

1.4 DNA Mismatch Repair and Meiotic Checkpoint System……...……17

1.5 Thesis Project…….19

2 EXPERIMENTAL SECTION…….21

2.1 Materials…….21

2.1.1 Strains and culture media……21

2.1.2 Chemicals and particles……..……..……..22

2.2 General Methods and Equipments……...22

2.2.1 Transformation by Biolistic bombardment….……23

2.2.2 Somatic MAC gene knockouts……..…….29

2.2.3 Germline MIC gene knockouts……...30

2.2.4 Verification of the knockouts…….33

2.2.5 DAPI staining and microscopy…….……..34

2.2.6 2-AP and MMS treatments…….35

3 RESULTS……...36

3.1 Phenotypes of the *Msh2* MAC Knockouts in *T. thermophila*…….36

3.1.1 The *Msh2* MAC knockouts.……36

3.1.2 The conjugation of an *Msh2* knockout cell and a wild type cell……....38

3.1.3 The conjugation of two *Msh2* knockout cells…….42

3.1.4 Cell pairing levels of the conjugations……...45

3.1.5 The MICs of the separated *Msh2* knockout cells after pairing……...46

3.1.6 Potential germline aging of the *Msh2* knockouts……..……..49

3.2 Phenotypes of the 2-AP or MMS Treated *Msh2* MAC Knockouts in *T. thermophila*…….50

3.2.1 Chemical mutagens 2-AP and MMS introduce mutations to the chromosomes and are the sources of genotoxic stress……....……...50

3.2.2 The conjugation of 2-AP or MMS treated wild type cells with different mating types……..……..51

3.2.3 The conjugation of a 2-AP or MMS treated *msh2* knockout cell and a non-treated wild type cell……...52

3.3 The *Msh2* MIC Knockouts in *T. thermophila*……..57

3.4 Phenotypes of the *Mlh1* Mac Knockouts in *T. thermophila*……...60

3.4.1 The *Mlh1* MAC knockouts……60

3.4.2 The conjugation of the *Mlh1* KO X WT and the *Mlh1* KO X KO…..……61

4 DISCUSSION……..……...65

4.1 Msh2 Is Indispensible for the Formation of Crescent Micronuclei……..65

4.2 Msh2 Protein May Be Involved In the Meiotic Intra-S phase Checkpoint……..70

4.3 Whether MSH2 and MLH1 Function In the Same MMR Pathway Remain To Be Confirmed……...73

4.4 MSH2 And MLH1 Affect the Cell Pairing of *T. thermophila*……76

5 FUTURE DIRECTIOS……..…….79

6 REFERENCES……...82

7 APPENDIX……...……..89

List of Tables

Table 3.1 Levels of cell pairing efficiencies during conjugations of WT and msh2 KO strains…….45

Table 3.2 Levels of cell pairing efficiencies during conjugations of non-treated WT and msh2 KO strains…….57

List of Figures

Figure 1.1: Events of *T. thermophila* cell conjugation……...……...16

Figure 2.1: Transformation assortment……...……...28

Figure 2.2: Micronuclear knockout process……...……31

Figure 3.1: mRNA RT-PCR of the msh2 knockouts…...……..37

Figure 3.2 A: The conjugation of two wild type cells with different mating types, and a MAC msh2 KO cell and a wild type cell with different mating types, 3h-6h of mix……40

Figure 3.2 B: The conjugation of two wild type cells with different mating types, and a MAC msh2 KO cell and a wild type cell with different mating types, 7h-10h of mix…..41

Figure 3.3 A: The conjugation of two wild type cells with different mating types, and two MAC msh2 KO cells with different mating types, 3h-6h of mix……..43

Figure 3.3 B: The conjugation of two wild type cells with different mating types, and two MAC msh2 KO cells with different mating types, 7h-10h of mix……44

Figure 3.4: Conjugation between the separated msh2 KO cell and the WT cell with a third mating type…….48

Figure 3.5 A: The conjugation of two wild type cells with different mating types 3h-6h, that were non-treated, 2-AP treated, and MMS treated…….53

Figure 3.5 B: The conjugation of two wild type cells with different mating types 7h-10h, that were non-treated, 2-AP treated, and MMS treated…….54

Figure 3.6 A: The conjugation between an msh2 KO cell non-treated, 2-AP treated, and MMS treated, and a non-treated wild type cell, 3h-6h……..55

Figure 3.6 B: The conjugation between an msh2 KO cell non-treated, 2-AP treated, and MMS treated, and a non-treated wild type cell, 7h-10h……56

Figure 3.7: mRNA RT-PCR of the MIC msh2 KOs……..59

Figure 3.8 A: The cell conjugation of WT X WT, mlh1 KO X WT, mlh1 KO X KO, 3h-6h……63

Figure 3.8 A: The cell conjugation of WT X WT, mlh1 KO X WT, mlh1 KO X KO, 7h-10h……..64

Figure 4.1: The theory of the formation of the crescent MIC……68

Figure 4.2: Expression of MMR genes in conjugating cells relative to vegetative cells...69

Figure 4.3: PMS2 protein localization……...74

Figure 5.1: Rescue experiments of the msh2 MAC KOs……...81

Figure 7.1: Restriction analysis of pKOCDS1……...91

List of Abbreviations

WT	Wildtype
KO	Knockout
MAC	Macronuclear/Macronucleus
MIC	Micronuclear/Micronucleus
MSH2	MutS Homologue 2
MLH1	MutL Homologue 1
PMS2	Postmeiotic segregation 2
IESs	Internal Eliminated Sequences
2-AP	2-Amino Purine
MMS	Methyl Methanesulfonate
MNNG	N-Methyl-N'-nitro-N-nitrosoguanidine
Chx	Cycloheximide
Pm	Paromomycin
6mp	6-methylpurine
ATM	Ataxia Telangiectasia Mutated
ATR	ATM and Rad3-related

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1 INTRODUCTION

The genome size of a prokaryotic organism is less than 10^7 base pairs. Eukaryotic genomes have bigger sizes. The human genome contains about a thousand times as many base pairs and twenty times as many genes as a bacterium. Genomic DNA is replicated during vegetative growth in cells and the replication process is complicated. Although eukaryotic genomes are huge, there are multiple origins that start DNA replication and the whole replication process is very fast. DNA replication is a semi-conservative mechanism. The newly synthesized DNA strand base-pairs to the template strand. During this base-pairing, errors happen and result in mis-pairing, like base substitutions, insertion or deletion loops, etc. Different forms of correction device maintain genomic fidelity step by step during DNA synthesis. First, base pair geometry elevates the fidelity to 10^{-4}-10^{-5} per base pair per replication. Second, DNA polymerase and exonuclease proofread the replication product and reduce the mistakes 100-1000 fold. The third attempt at correction is realized by the mismatch repair (MMR) system, which corrects the replication errors that have escaped from the proofreading step. If the errors are still uncorrected, they will be fixed during the next round of DNA replication as

mutations. Finally, the spontaneous mutation rate is 10^{-9}-10^{-10} per base pair per replication (Drake, 1998; Iyer, 2006 review).

Mismatches can also be derived from genetic recombination during meiosis. Before cells undergo meiosis, chromosomes are duplicated to form sister chromatids. Each of the two chromatids can cross over with either of two chromatids of other chromosomes. Crossovers can cause mismatches when they happen between DNA strands through base pairing.

DNA mismatch repair system corrects replication errors and genetic recombination errors. It also processes base pair anomalies caused by DNA damaging agents. The mechanism of mismatch repair was well studied in *E. coli* (Yang, 2000 review; Iyer, 2006 review) during their mitotic state. The mismatch repair system is believed to repair base-base and insertion/deletion loop mismatches. It is a methylation-dependent process. MMR enzymes include MutS, MutH, MutL proteins and other MMR-related proteins. MutS and MutL proteins function as homodimers. MutS recognize mismatches and MutL mediate the links between MutS and MutL, and other MMR-related proteins. MutH is an endonuclease that nicks the DNA strand at a hemi-methylated site. UvrD removes the strand from the nick to the mismatches; it leaves a single gap in the

DNA. Then, DNA polymerase binds to the single strand and the gap is repaired using the complementary strand as a template. However, the mechanism of mismatch repair in eukaryotic organisms is still unclear (Iyer, 2006 review). No MutH homologue has been identified in eukaryotic cells. MutL has part of MutH's function as an endonuclease (Yang, 2007). MutS and MutL homologues function as heterodimers. Eukaryotic organisms use similar MutS and MutL homologues as MMR enzymes. This will be explained later in this introduction.

Mismatch repair systems have been studied in some eukaryotic microorganisms, like yeasts and humans. Here in this thesis, a fresh-water ciliated protozoan, *Tetrahymena thermophila*, was used as a model to study the functions of MMR protein homologues during meiosis. This ciliate has been identified as a good model to study DNA deletion and rearrangement (Yao, 2005 review) and telomere formation (Fan, 1996) based on its special characteristics. *T. thermophila* is a single cell organism with two nuclei, a micronucleus (MIC) and a macronucleus (MAC). The MIC is a germ-line nucleus that is transcriptionally silent; the MAC controls somatic behaviors and is transcriptionally active. *Tetrahymena* cell is a big cell with 40-50 micrometers in length along the anterior-posterior axis, which is about 10 times bigger than a yeast cell. It

contains a huge amount of genome, 220 mega base pairs, which is of the same order of magnitude as that of Drosophila, one order larger than yeast, and one order smaller than human. *T. thermophila* reproduces by binary fission and conjugates between two different mating types. The conjugation is inducible, which is an advantage to study protein functions during meiosis in *T. thermophila*.

1.1 DNA Mismatch Repair System

As we know, the spontaneous mutation rate from the DNA replication process is very low; the MMR pathway is the last fidelity service to maintain genomic integrity, and elevates the fidelity 100-1000 folds. In this pathway, base substitutions, mismatched loops and some DNA damage can be repaired. Defects of the mismatch repair system in humans are correlated with predisposition to hereditary non-polyposis colorectal cancer (Froggatt, 1996; Jover, 2004). Error-free genetic recombination is guaranteed and DNA exchange between species is inhibited by the MMR system. DNA mismatch repair pathway and other repair

pathways, like the nucleotide excision pathway (Wu, 2005) and very short repair pathway (Monastiriakos, 2004), interact and function coinstantaneously for DNA repair.

1.1.1 Mechanism of mismatch repair

The process of mismatch repair is composed of sensing (detecting), signaling, recruiting and correcting. The mechanism of mismatch repair in *E. coli* is explained as follow. As mentioned before, Mut proteins and some DNA synthesis enzymes are required in the process.

Sensing or **Detecting** G-T (Lamers, 2000), A-C, G-A, T-C, A-A, G-G, T-T mismatches and 1-4 nucleotide insertion/deletion loops (Su, 1986) can be recognized by MutS protein; C-C is the only mismatch that is not well repaired (Iyer, 2006 review). MutS protein has a mismatch-dependent ATPase activity (Haber, 1991) that makes it function as a damage sensor (Acharya, 2003). The homodimer of MutS forms a clamp and binds to the DNA substrate. DNA binding site and ATPase centers of MutS interact strongly which proves that it is DNA that activates ATP hydrolysis (Iyer, 2006 review).

Signaling *E. coli* mismatch repair is methylation-dependent and strand-specific. The newly synthesized strand may have replication errors. The template (parental) strand is methylated at GATC sequences and the daughter strand is not. This hemi-methylation process signals DNA strand cleavage by MutH protein. Thus the mismatch repair is initiated (Iyer, 2006 review).

Recruiting MutL proteins are recruited by MutS (Iyer, 2006 review; Yang, 2000 and function as a homodimer. MutL protein mediates the initiation of mismatch repair by interacting with both the MutS and MutH proteins. MutH is a GATC endonuclease. MutS, MutL, and ATP are requird for MutH activation. MutH nicks the methylated strand at the nearest GATC site and recruits UvrD helicase to separate and unwind the DNA strands.

Correcting MutS-MutL-MutH-UvrD complex slides along the strand until the site of mismatch. An exonuclease digests the single-strand DNA and creates a gap, which is then repaired by DNA polymerase III and sealed by DNA ligase. Finally, the corrected daughter strand is methylated immediately for the next cycle of DNA replication.

1.1.2 Eukaryotic mismatch repair enzymes

Mut proteins are highly conserved in eukaryotic organisms. However, no eukaryotic MutH homologue has been identified. The signals that direct mismatch repair in eukaryotic cells are unclear.

There are multiple MutS and MutL homologues in eukaryotic organisms, which probably explains why mismatch repair system is more complicated. MutS and MutL homologues function as heterodimers in eukaryotes, including α and β heterodimers, composed of different MMR proteins. For MutS homologues, six homologues have been designated as MSH1-MSH6. MSH1 plays a role in mitochondrial DNA stability (Reenan, 1992). MSH2-MSH6 dimer forms MutS-α, and MSH2-MSH3 dimer forms MutS-β. MSH4-MSH5 dimer is restricted to meiosis and has no mismatch repair function. For MutL homologues, in yeast, there are MLH1-PMS2 (MutL-α) dimer, MLH1-MLH2 dimer and MLH1-MLH3 dimer. In human, there are MLH1-PMS2 (MutL-α) dimer and MLH1-PMS1 (MutL-β) dimer. Thus, MSH2 and MLH1 proteins are essential for eukaryotic mismatch repair system.

Human MutL-α has a probable endonuclease active site in a motif of PMS2 subunit (Kadyrov, 2006), which may resemble the MutH excision mode in *E. coli* mismatch repair system (Yang, 2007).

As mentioned in *E. coli* mismatch repair system, UvrD helicase unwinds the nicked strand. However, no DNA helicase has been identified in eukaryotic MMR system. DNA polymerase δ, replication protein A (RPA, DNA binding protein), proliferating cell nuclear antigen (PCNA, the replication sliding clamp) and replication factor A (RFA) are required in human MMR (Genschel, 2006; Iyer, 2006 review). Both MutS-α and MutS-β dimers have a PCNA recognition motif that is located near the N-terminus of MSH6 and MSH3 subunits respectively in human (Kleczkowska, 2001).

In *T. thermophila*, multiple Mut protein homologues have been identified. For MutS homologues, there are one MSH2 homologue and four MSH6 homologues; no MSH4 or MSH5 homologue is known so far. For MutL homologues, there are MLH1 and PMS2 homologues.

1.2 *Tetrahymena thermophila* Model

Tetrahymena thermophila is one of the most characterized species of ciliated protozoan. It was considered to be a member of *the Tetrahymena pyriformis* group at first, because the two species of ciliates display the same morphology. *T. thermophila* is a fresh water microorganism in nature. It has animal-like behaviors, "swimming" in the water for example. The oral apparatus at the anterior end of the cell detects surrounding nutrient environment and contacts other individuals. As a laboratory model organism, *T. thermophila* has many advantages. It can be cultured in diversified media at a wide range of growing temperatures. Its doubling time is 2-2.5 hours in 2% SSP medium at 30°C. Cell conjugation between two different mating types is inducible, and pairing efficiency above 95% can be obtained.

T. thermophila has a large evolutionary distance from other laboratory used eukaryotes. Its significant nuclear dimorphism makes it to be a good choice in some special research fields. In this thesis, *T. thermophila*'s large cell size is advantageous for microscopic imaging to observe cells during meiosis.

Nuclear dimorphism *T. thermophila* is a single cell microorganism with two nuclei. The two nuclei have clearly differentiated structures and non-overlapping functions. However, amicronucleate *T. thermophila* exist commonly in nature.

1.2.1 Macronucleus (MAC) of *T. thermophila*

MAC is the somatic nucleus in *T. thermophila*. Its genome size is 104 mega base pairs (Eisen, 2006). MAC has about 45 copies of the haploid genome and is transcriptionally active. Two of the three stop codons used in the universal genetic code, UAA and UAG, encode glutamine instead, leaving only one stop codon available in *T. thermophila*. The reassigned protein coding makes some commonly used protein expression systems inaccessible to express *T. thermophila* proteins. MAC divides amitotically; about half of the DNA is distributed into each of the two daughter cells.

(New) MAC development The genome of MAC is derived from MIC during MAC differentiation. The development process involves programmed genomic DNA deletion and rearrangement, which was reviewed by Yao, 2005. Deletion elements or internal eliminated sequences (IESs) are MIC-specific (limited) and

not obvious protein-coding sequences. They are spliced out at more than 6000 sites throughout the chromosomes, resulting in different sizes of DNA pieces, named autonomously replicating pieces (ARPs), ranging from several hundred baspairs to more than 10 kilo basepairs. These ARPs are amplified to about 45 copies, and developed to a new MAC genome. The deleted DNA makes up to about 15% of the total genome (Yao, 2005 review).

Phenotypic assortment MAC is transcriptionally active and genes are expressed in this nucleus. As mentioned before, MAC divides amitotically, the distribution of the genetic material into each of the two daughter cells may not be done equally. MAC controls the phenotype of the cells and produces different phenotypes. Before cell division, the newly developed MAC genome from heterozygous MIC contains equal amount of two alleles; however, the distribution of the alleles into each of the two daughter cells during division may be unequal. It is possible that all copies of one of the two alleles can be lost in some cells, and these cells appear to be pure for the other allele (Merriam, 1988).

Sexual maturity Successful progeny need to experience 65 generations of intermediate stage and 20-25 generations of adolescence stage to become sexually mature again (Rogers, 1985). At intermediate stage, cells are immature

and unable to mate with other individuals. At adolescence state, cells can mate with other mature ones but not with adolescence state or younger state ones. When cells become sexually mature again, mating ability is recovered between any two different mating types.

1.2.2 Micronucleus (MIC) of *T. thermophila*

MIC is the germline nucleus in *T. thermophila,* and is composed of 5 diploid chromosomes. It is transcriptionally silent and responsible for storing genetic information; very few genes are expressed in the MIC. MIC divides mitotically during vegetative growth, and undergoes meiosis during cell conjugation.

The *Tetrahymena* strains that have no contributable MICs are named "star" strains. When a cell with a functional MIC (cell A) conjugate with a cell of a star strain (cell B), **genomic exclusion** is induced. Genomic exclusion includes two rounds. In round I, once cell A and cell B pair together, the MIC in cell A undergoes mitosis and cell B lose its defective MIC. One of the mitotic products is transferred to cell B. MICs in both cell A and B diploidize to form diploid MICs. When the cells are left in conjugation medium, they will enter round II

immediately. In round II, diploid MICs undergo mitosis, and only one of the four mitotic products is maintained. The new MACs are derived from the MIC. Four identical progeny are formed finally (Hai, 1997).

1.3 *Tetrahymena thermophila* Meiosis

There are 7 different mating types in *T. thermophila*, any two of them can be induced to conjugate in proper condition. Conjugation is the sexual stage of the life cycle in *T. thermophila*. No cell division happens. During conjugation, cells undergo a series of events, like forming a temporary junction, exchanging gamete nuclei and differentiating the nuclear apparatus of their sexual progenys, etc. Before the cells can conjugate properly at high pairing efficiencies, some requirements should be satisfied. Cells must, first, be starved for at least 8 hours; second, be of different mating types; third, be sexually mature (explained before).

Starvation-mediated development While the cells are starved in starvation buffer (10 mM Tris-HCl, pH 7.4) at 30°C in a moist chamber, they detect the surrounding nutrient environment and do self-development to prepare for

conjugation. The developmental process includes 1) initiation, 2) co-stimulation; cells decide to pair with other initiated partners depending on cell contact, and 3) pre-meiotic DNA replication; cells duplicate their DNA before they enter conjugation.

Cell conjugation in *T. thermophila* Once the cells pair, the MICs move out from their MAC pockets and become visible (Figure 1.1 A). (MIC is nested in the MAC in non-dividing cells, which makes it invisible sometimes under the microscope. However, MIC and MAC are physically separate.) MIC elongates to form a crescent phase, lacing around the MAC (Figure 1.1 B-C); its length can increase by 50-fold (Wolfe, 1976). Following the crescent phase, MIC chromosomes condense and undergo meiosis I and II (Figure 1.1 D). One of the four meiotic products is selected (Figure 1.1 E) for pre-zygotic mitosis (Figure 1.1 F), and the other three are left to degrade. Two identical pronuclei are produced, one stationary pronucleus and one migratory pronucleus. The stationary ones remains in the original cells and the migratory ones are exchanged between conjugating partners (Figure 1.1 G). The two pronuclei in each cell fuse to form the fertilization nucleus (Figure 1.1 H) that is the progenitor of both the new MIC and new MAC. Following the nuclear fusion, the cross-fertilized zygote nuclei

undergo post-zygotic division twice (Figure 1.1 I), and products localize differently. The two anterior localizing nuclei develop into new MACs; the two posterior localizing nuclei form the new MICs; the cells reach MAC anlagen phase. In anlagen II (Figure 1.1 J), the old MAC moves to the posterior of the cell and undergoes apoptosis (Figure 1.1 K). The paired cells separate. One of the new MICs in each individual is degraded and the other divides mitotically. Finally, four progeny of the conjugated cells are produced (Figure 1.1 L). The progeny need to experience about 90 generations to become sexual mature again.

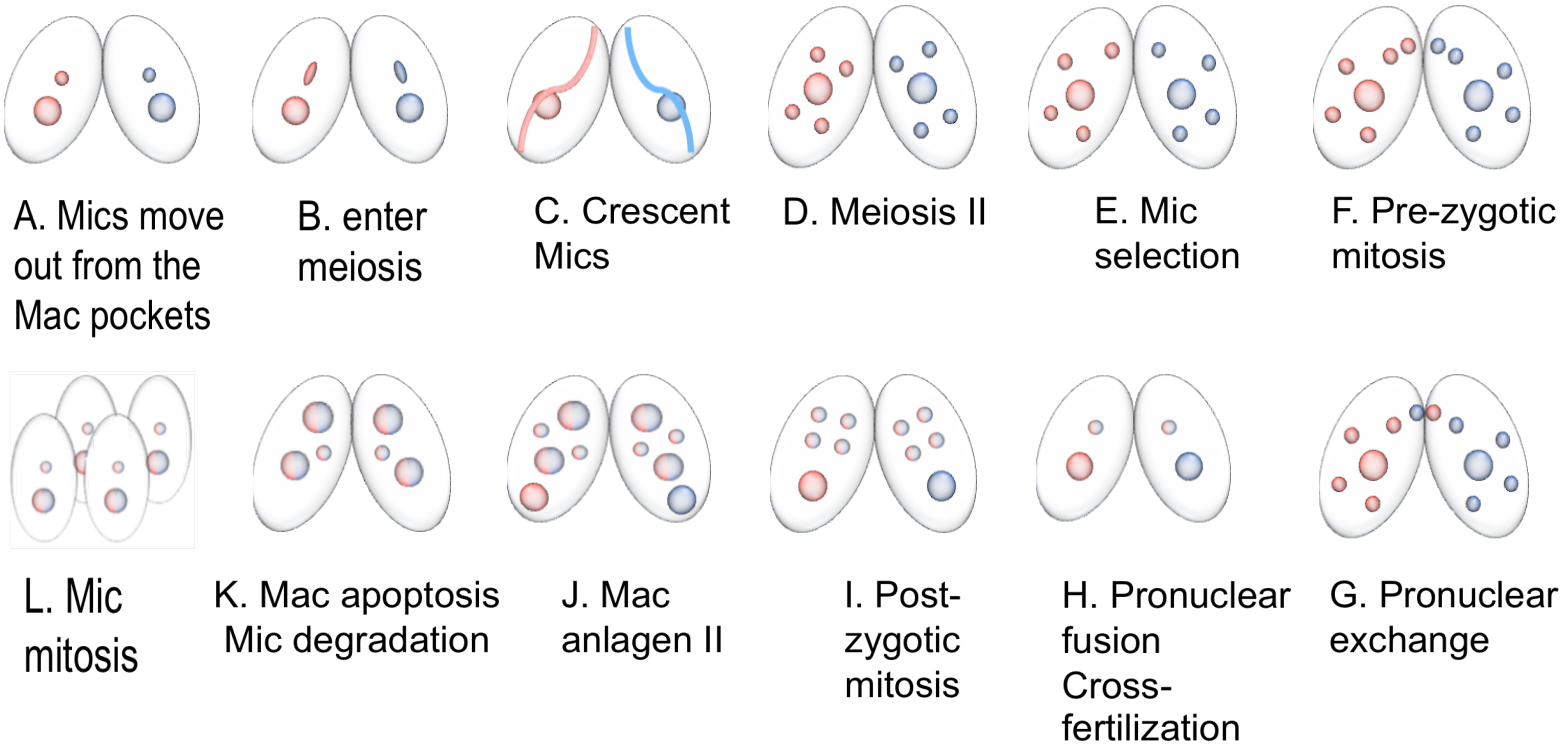


Figure 1.1 Events of *T. thermophila* cell conjugation.

1.4DNA Mismatch Repair and Meiotic Checkpoint System

MMR elevates DNA replication fidelity. Checkpoint systems maintain genomic integrity within the cell cycle. If a certain level of DNA damage has been accumulated from the replication process or from DNA damaging agents, the cell cycle will be arrested to allow checking and repairing of the damage. Cells follow G1-S-G2-M cycles. “G” means Gap phrase, “S” means Synthesis of DNA, and “M” means Mitosis in a mitotic cell cycle. There are mitotic checkpoints and meiotic checkpoints. However, the mechanism of meiotic checkpoint systems is still uncertain. In mitosis, there are G1/S, intra-S phase and G2/M checkpoints. G1/S checkpoint guarantees the cells are well prepared to enter S phase. Intra-S phase checks the fidelity of synthesized DNA. G2/M checkpoint guarantees the cells are well prepared to enter M phase.

For instance, the G2/M checkpoint system, that is reviewed by O’Brien, 2006. ATM, CHK1 and CHK2 checkpoint enzymes are required (O’Brien, 2006 review; Adamson, 2005). Methylating / alkylating agents like MNNG and MMS, trigger MMR-dependent G2/M cell cycle arrest. This arrest requires ATM/ATR to phosphorylate its effector CHK1 checkpoint kinase. MutLα (MLH1) interacts with

ATM; MutSα (MSH2) interacts with ATR (Wang, 2003) and CHK2. These interactions are enhanced in response to DNA damage (Brown, 2003). CDC2 is the final target in the replication and repair checkpoint (Murakami, 2000). CDC2 induces cell mitosis, and is activated by a phosphatase CDC25. This activation can be regulated by CHK1 that phosphorylates and inhibits CDC25. Wee1 can inhibit CDC2, which is regulated by CDS1. The replication checkpoint requires the inhibitory phosphorylation of CDC2. CHK1 and CDS1 appear to jointly enforce the replication checkpoint.

CDS1 is a protein kinase that is believed to play a more significant role during meiosis than it does during mitosis in fission yeast (Murakami, 1999). In a meiotic DNA replication checkpoint system, CDC2 is phosphorylated in order to maintain its low activity. Thus, the cell cycle is blocked and meiosis I is inhibited.

MEK1 is a meiosis-specific kinase that belongs to a kinase family, the CDS1/RAD53/CHK2 family. MEK1 has a role in the meiotic recombination checkpoint in fission yeast (Perez-Hidalgo, 2003). CDC25 phosphatase can be inhibited by MEK1 because of the dephosphorylation of CDC2. MEK1 prevents the cell from entering meiosis I until the recombination is complete.

1.5Thesis Project

T. thermophila is a laboratory model organism which has a large somatic macronucleus and a small germline micronucleus that have non-overlapping functions but exit in a single cell at the same time. The protein localization and nuclear morphology can be observed easily in the huge single cell organism, which makes *Tetrahymena* a good tool to study the potential interactions between mismatch repair proteins and nuclei. In addition, *Tetrahymena*'s special sexual habits and events make it a good model to study the roles of the mismatch repair proteins during meiosis.

MSH2 and MLH1 proteins are essential MutS and MutL homologues in eukaryotic MMR systems respectively. MSH2 protein recognizes replication or recombination errors such as those that lead to base pair substitution and frame-shift mutations in human and yeast (mentioned before). MLH1 protein in eukaryotic cells interacts with MSH2 and may have a similar role of endonuclease. These two proteins are believed to be MMR proteins; MSH2- or MLH1- cells seem to be able to tolerate more DNA damages. Humans that have MMR defects have much higher chance to form colon cancer.

The purpose of this project is to study the roles of the MSH2 and MLH1 proteins during meiosis in *Tetrahymena* by morphology. It is hypothesized that these two proteins have effects during the cell conjugation because of the defects of the MMR system.

Molecular genetic techniques were used in *Tetrahymena* to study the MMR gene functions in vivo, such as DNA-mediated transformation, gene knock-out by homologous recombination, epitope (GFP) tagging and inducible gene expression. Msh2 and Mlh1 complete knockouts were made in this project to study the gene functions in *T. thermophila*.

2 EXPERIMENTAL SECTION

2.1 Materials

2.1.1 Strains and culture media

Tetrahymena thermophila wild type strains CU427 (VI), CU428 (VII), B2086 (II), and star strains A* (V), B* (VI&VII) were used for this research. Genotype and phenotype for genetically marked strains are: CU427: chx1-1/chx1-1 (cy-s, VI); CU428: mpr1-1/mpr1-1(mp-s, VII).

Cells were routinely cultured in 2% SSP, a rich axenic nutrient medium. It contains 2% proteose peptone (PP), 0.1% yeast extract, 0.2% glucose and 0.003% Fe-EDTA. In this medium, the doubling time of cells is around 2.5h at 30°C with aeration.

Starvation medium contains 10mM Tris HCl, pH 7.4-7.5. This medium is required to starve *Tetrahymena* cells for many experimental procedures. Starvation was realized in starvation medium at 30°C after at least 8 hours, shaking or not, (usually over night); and the cells develop their sexual

competence before mixing. The starved cells can remain alive for about 2 days. Before cell conjugation, equal numbers of cells with any two different mating types were mixed well at 30 °C. At this time, the cells are sensitive to temperature, which can affect the cell pairing efficiency. After mixing, wild type cell pairing efficiency can reach to above 90% in 2 hours, at °C without shaking. Shaking condition may separate paired cells, although it is good for cell growth in *T. thermophila*.

2.1.2 Chemicals and particles

Paramomycin (SIGMA), 6-methypurine (SIGMA), cycloheximide (SIGMA), CdCl2 (SIGMA) and 2-aminopurine (SIGMA) are dissolved in sterile distilled water at a final concentration of 200 μg / mL, 15 μg / mL, 25 μg / mL, 1 mg / mL and 10 mg / mL respectively, then sterilized by syringe filter. MMS purum (SIGMA) is dissolved in 10% DMSO, ≥98.0% (GC). Gold microparticles (BIO-RAD) were sterilized by 70% ethanol and resuspended in sterile 50% glycerol to a final concentration of 60 mg / mL.

2.2 General Methods and Equipment

2.2.1 Transformation by Biolistic bombardment

Each vegetative transformation needs a total of 1 x 10^7 cells. Each germline transformation needs 5 x 10^6 cells for each of the two different mating types. The cells were grown up and starved (the same way as starvation mentioned above).

Mating pairs are required for germline micronuclear or developing macronuclear anlagen transformation. Cells of two different mating types were mixed in equal numbers and incubated without shaking at 30°C. To optimize mating, the depth of the mating cultures was kept as shallow as possible to facilitate gas. At 2h and 3h after mixing, the mating was checked by placing one drop of the cell suspension on a clean slide under the microscope. The efficiency of the mating was calculated roughly. If the mating efficiency was above 75% at 3h post mixing, the particle coating can be prepared for the bombardment. Since the number of pairs in the appropriate stage of mating limits the efficiency of germline transformation, care should be taken to optimize efficiency of pairing.

Particle coating. 25µl of the microparticles were taken from the gold beads stock into a 1.5 ml microfuge tube. Solutions were added to the tube with the particles while vortexing the tube, in this order: 4µg DNA, 25µl 2.5 M cold CaCl2,

10µl 0.1 M spermidine. After vortexing completely, the tube was taped to another vortex in a cold room and vortex for 10 min. Then the tube was centrifuged for 10 sec and the supernatant was discarded. The DNA-coated microparticles were washed with 200µl cold 70% ethanol and 100% ethanol, and the supernatant was pulsed and discarded. 20µl 100% ethanol were added to resuspend the DNA-coated microparticles which were mixed by pipette. The 20µl mix was spotted into the center of a flying disk, which was placed on the holder in a desiccator to dry the microparticles.

The Bio-Rad PDS-1000/He biolistic gun uses high pressures and the rupture disk-microcarrier distance, the microcarrier travel distance, and the distance between the stopping screen and the biological target must all be adjusted to maximize transformation according to recommendations of the manufacturer.

Particle bombardment. The starved vegetative or mating cells were spun down at 1100 X g and resuspened in 250µl Tris buffer. Then the cells were spread onto the center of a pre-wetted 70mm filter paper in a Petri dish. The biolistic gun fired the DNA-coated microparticles into the cells, and the bombardment was performed according to recommendations of the manufacturer.

The time periods for the two kinds of transformation are very narrow. For germline transformation, the bombardment should be done between 2.75 and 4.5 h (at crescent phase) after mixing pre-starved cells; bombardment at 3.5 h after mixing shows the highest transformation efficiency (Cassidy-Hanley, 1997). For transformation of developing macronuclear anlagen, the bombardment could be done between 9.5 and 10.5 h after cell mixing (anlagen development). For optimum results, the nuclear morphology of DAPI stained cells is required to check to ensure that the cells are in the correct developmental stage. In addition, the pathway is extremely temperature-sensitive, and the timings listed here are for cultures kept at 30°C for all steps except the shooting.

After bombardment, the filter paper was rolled and placed into a pre-warmed flask with 50 ml growth medium, shaking gently for about 4h at 30°C. The two most common selectable markers, conferring resistance to paromomycin and cycloheximide respectively, require drug additions at different times. Paromomycin resistance is conferred by the bacterial transposon-derived aminoglycosidase 3' phosphotransferase gene aph (Kahn, 1993). The *Tetrahymena*-derived gene *RPL29A* encodes a cycloheximide-resistant mutant version of a ribosomal small subunit protein homologous to the product the yeast

Cyh2 locus (Yao, 1991). In order to select successful transformants, paromomycin at a starting concentration of 50 μg / mL was added to the culture after 4h of recovery. The cells were aliquotted to 96-well plates and cultured in a moisture chamber at 30°C. Clones of resistant transformants (blooms) could be seen after 3 or 4 days and kept coming up for several more days.

The concentration of paromomycin was increased step by step (Figure 2.1) to assort complete knockouts, and could be pushed to at least 7 mg / mL. Complete knockouts were verified by genomic PCR and RT-PCR.

For cycloheximide selection, after the biolistic shoot, cells were resuspened in 1 x (1%) SSP with 1 μg / mL $CdCl_2$ to induce the expression of the *RPL* gene by the MTT promoter. $CdCl_2$ was maintained in the medium until knockout verification. After growing in that medium over night at 30°C, cycloheximide was added to the medium at a starting concentration of 15 μg / mL. The concentration of cycloheximide can be pushed to 200μg / mL to assort complete knockouts.

Mating cells were allowed to complete the conjugation and initiate vegetative growth. After the biolistic shoot, mating cells were resuspended in pre-warmed Tris buffer and incubated at 30°C for 17-20 h. After the cells completed

conjugation, they were spun down and resuspend in 2% SPP growth medium. Then the transformantas were selected as described above.

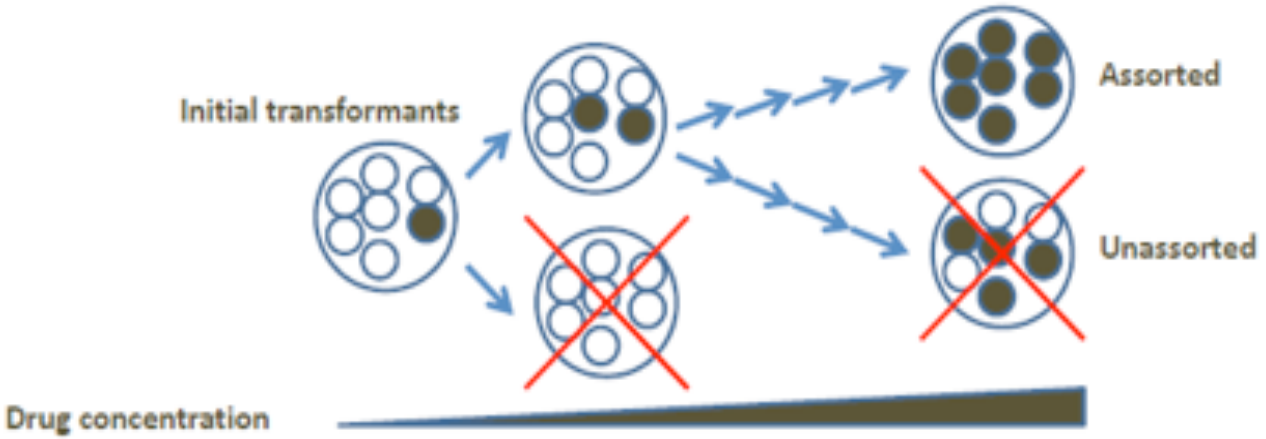


Figure 2.1 Transformant assortment. After biolistic transformation, the initial transformants were selected by paromomycine (50 ug/mL). With the increasing of the concentration of the drug, the cells with more copies of msh2 gene replacement could survive. Drug concentration was increased until the whole 45 copies (approximately) of msh gene were replaced by the *neo* cassette in the genome. Unassorted cells died at high drug concentration that they could not endure.

2.2.2 Somatic MAC gene knockouts

As mentioned in the Introductionpart, the macronucleus of *T. thermophila* is transcriptionally active and all known genes are expressed in the macronucleus. In order to study phenotypes of *Tetrahymena* MMR genes, essential MMR genes were knocked out in the somatic macronucleus in this project. *Msh2* and *Mlh1* genes were knocked out completely, and each of them was done in cells with at least two different mating types.

The vectors used to make *MMR* gene MAC knockouts were made previously by cloning PCR products corresponding to sequences 5' and 3' of the target gene on either side of the selectable *neo* cassette in pH4T-1. In the *neo* cassette, there is a 300bp H4 promoter and a 300bp 3' region of BTU (Gaertig, 1992; Gaertig, 1994). Following transformation and recovery, cells were exposed to gradually increasing concentrations of drugs. Target gene was replaced copy by copy by phenotypic assortment until all the ~45 ARP copies of a target gene in the macronucleus were replaced.

The knockouts of CDS1 (a major kinase in checkpoint system, Chk2 in human) in *T. thermophila* have been done in the same way as the knockouts of MMR

proteins. The vector used to make *CDS1* MAC knockouts was made in this project based on vector pMrpL29B (from Dr. Gorovsky's lab). Different selectable markers were used in this knockout. 5' and 3' flanks of *CDS1* were amplified by PCR using primers:

5'flankF1: 5'(*Xba*I)GCTCTAGAGCCACCGTGATGTTTCTCACTCATGTG3';

5'flankR1: 5'(*Spe*I)GGACTAGTCCCTCAATCTGCTTAGCTATCTGCAT3';

3'flankF2:5'(*Xho*I)CCGCTCGAGCGGGATAGATCATACATAATAATCAAGCC3';

3'flankR2: 5'(*Kpn*I)CGGGGTACCCCGCTAGATTCATTCCTAATGAAAG3'.

2.2.3 Germline MIC gene knockouts

Germline gene knockout results in deletion of the target gene from both micronucleus and macronucleus *in Tetrahymena* cells. MIC knockouts are stable knockouts, which will not be lost after cell conjugation. The method is described as follows (Figure 2.2).

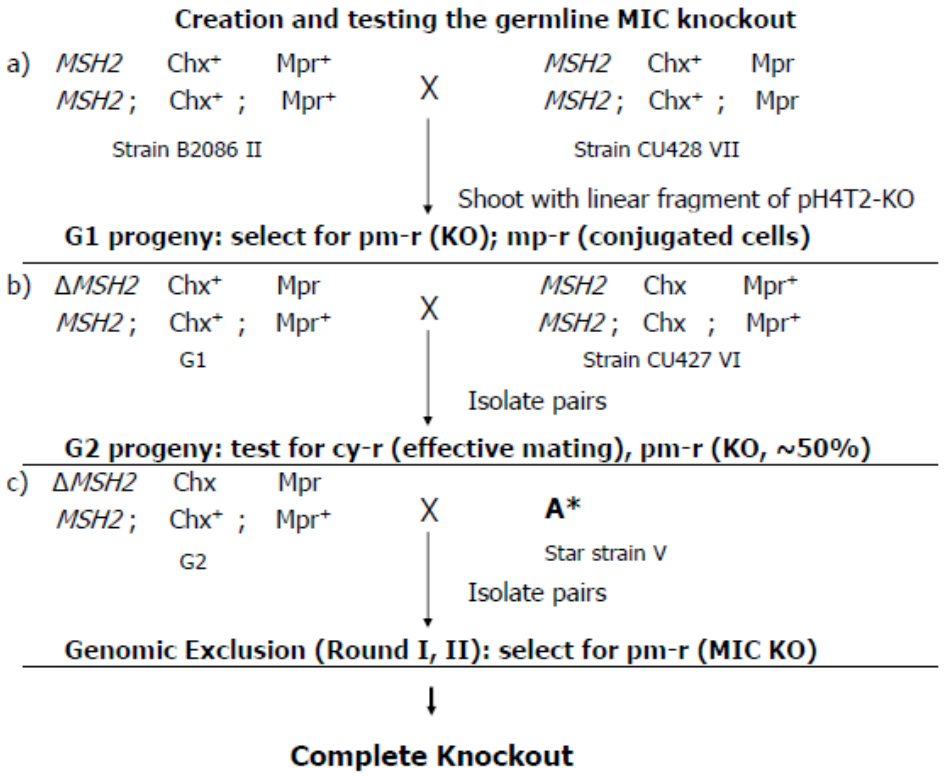


Figure 2.2 Micronuclear knockout process.

Cell conjugation is necessary for phenotypic selection to make micronuclear knockouts because micronuclei are transcriptionally silent. Cells that have just completed conjugation are immature and cannot mate again for about 90 fissions. The G1 transformants need to be mature again before they can be tested by a genetic crossing. The transformants were grown for at least 90 generations, and the genotype of G1 was determined by mating the cells to CU427 strain, which contains a cycloheximide resistance marker in the MIC. Synclones (G2) were first rested in cycloheximide to select successful mating and then in paromomycin to select for genetic transmission of the disrupted micronuclear gene. Cycloheximide-resistant G2 clones were expected to show 1:1 Mendelian segregation of the wild-type and the disrupted genes, therefore approximately 50% of cy-resistant G2 were expected to be also pm-resistant. After confirmation of the knockout in the micronucleus, G2 was set to make a star* strain through genomic exclusion (described in Introduction). The star strains had defective MICs that could not donate genetic material. The normal strain donated a gametic nucleus. The single haploid nucleus in each conjugate diploidized, and most cells separated without forming a new MAC. The final MIC knockouts were tested for paramomycin resistance and confirmed by crossing with CU427. Only a single germline transformation was needed to create knockouts of two different

mating types, because each transformed pair produces four karyonidal clones which are genetically identical but can have different mating types. If only a single mating type is obtained, a second one can be obtained by using another star strain with a different mating type.

2.2.4 Verification of the knockouts

Genomic DNA Isolation and PCR. This procedure is based on the protocol of Gaertig, 1994. Cells were grown to mid log phase (3-5 x 10^5 cells / ml) and starved in 10 mM Tris-HCl pH 7.4-7.5 for 18-24 h at 3 x 10^5 cells / ml. On the second day, starved cells were spin down at 1100 x g (Thermo Scientific CL40) for 5 min and resuspend in 3.5 ml urea buffer (8 M urea in 0.1 M phosphate buffer). When the lysate was homogeneous, it was transfered to a 15 ml Falcon tube and extracted twice with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and once with chloroform: isoamyl alcohol (24:1) at 3500 rpm. 1 ml 5 M NaCl was added to the approximately 3 ml of lysate recovered and DNA was precipitated with an equal volume of isopropyl alcohol, spooled onto a pipet tip, and transfered to a microfuge tube, washed with 70% ethanol. When the tube was dry, the DNA was resuspend in 600 µl TE buffer and 6 µl RNAse A (10 mg/ml)

was added. Then the tube was heated at 55°C over night. On third day, pelletable carbohydrates were removed by centrifugation at 35,000 rpm (Thermo Scientific CL40) for 45 min at 20°C.

Neo forward primer (5'-TGGCAAGCTTGGATGGATTGCAC-3') and a reverse primer in the 3' flank region (ordered previously in the lab) were used for the PCR confirmation of the KOs, which give a PCR product of 1.9kb.

Messenger RNA Isolation and RT-PCR. The messenger RNA was isolated by QIAGEN RNeasy Micro Kit. RT-PCR was done following QIAGEN OneStep RT-PCR Kit. F1 and R1 primers of *MSH2* (ordered previously in the lab) were used for the RT-PCR, which give a PCR product of about 600bp. 17S rDNA primers were used as the control, which gives a PCR product of about 300bp.

2.2.5 DAPI staining and microscopy

For staining the cells with DAPI, 40 µl of cells were spotted on a glass microscope slide and air-dried. The slides were soaked in jars with solutions 15 sec each in this order: 1) 95% EtOH, 2) DAPI stain (70% EtOH / 300 m M NaCl), 3) 70% EtOH, and 4) 35% EtOH. Cells were examined on a Leica DM6000B fluorescent microscope at 10x, 20x, and 40x magnification.

2.2.6 2-AP and MMS treatments

2-AP and MMS mutagens were used to induce mismatches and bring stress, which triggers the activation of the damage sensor. I used the amount of 2-AP or MMS which, when used to treat WT X WT gives similar conjugation efficiency to non-treated WT X WT. The concentration of the 2-AP is 250 μg / mL and the concentration of the MMS is 0.1%. When cells were grown up (2-5 x 10^5 / mL), mutagen was added into the medium. The cells were kept shaking for 1h and then the drug was washed off twice by Tris buffer. Cells were grown up again and starved until next day for conjugation induction.

3 RESULTS

3.1 Phenotypes of the Msh2 MAC Knockouts in *T. thermophila*

3.1.1 The msh2 MAC knockouts

In order to study the role the MSH2 protein during *Tetrahymena* cell conjugation, the *Msh2* gene has been completely knocked out in the macronuclei of three strains with different mating types in *T. thermophila*, CU427, CU428 and B2086. The complete msh2 knockouts were confirmed by genomic PCR and mRNA Reverse Transcriptase PCR (Figure 3.1). Knockout cells were grown up to $5x10^5$ cells / ml before the genomic DNA and messenger RNA were isolated. For the RT-PCR, in the KO cells, there are products from the reactions of the 17S rDNA primers but no product from the reactions of the msh2 primers; in the WT control, there are products from both of the reactions of the 17S rDNA primers and the msh2 primers, confirming complete gene deletion.

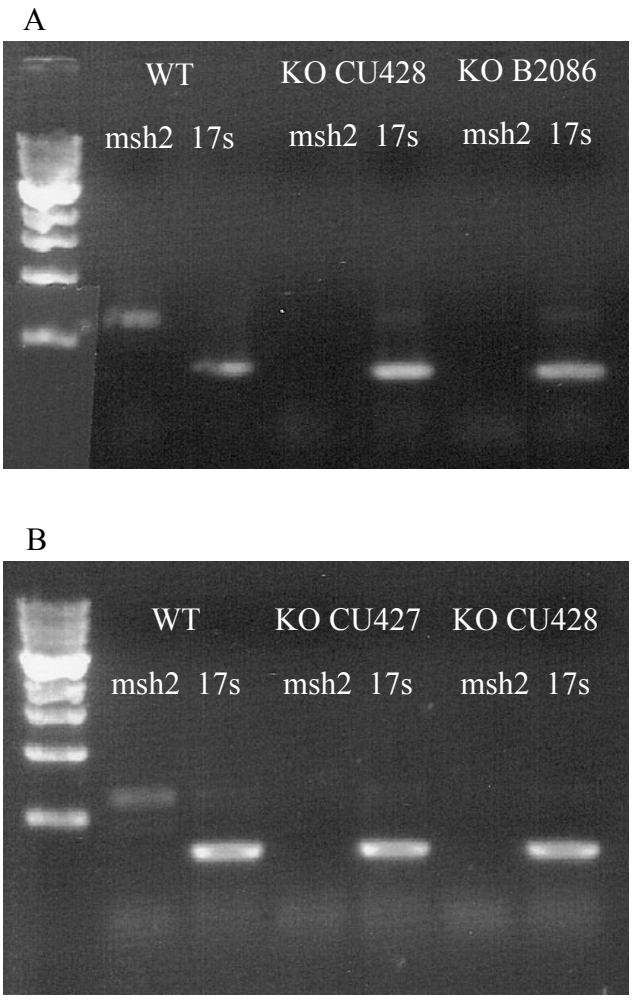


Figure 3.1: mRNA RT-PCR of the *msh2* knockouts.

A: *msh2* KO CU428 and B2086 (first time);

B: *msh2* KO CU427 and CU428 (second time).

3.1.2 The conjugation of an *Msh2* knockout cell and a wild type cell

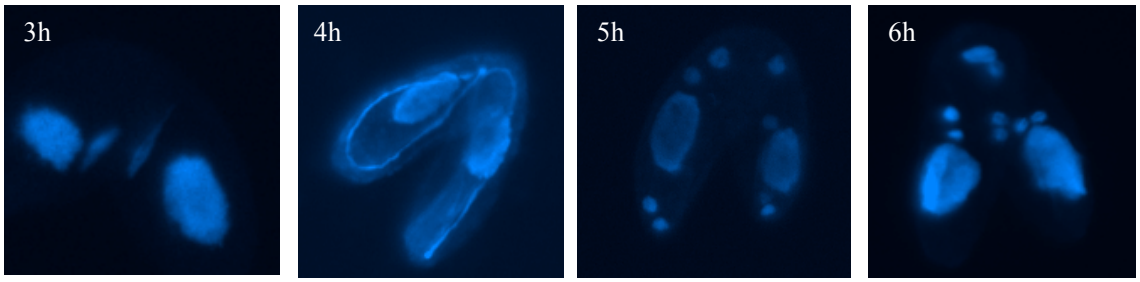
The conjugation between an msh2 knockout cell of one mating type and a wild type cell of another mating type was set up to study the phenotype of the KO cells. The WT X WT conjugation between different mating types was used as the control. 3h after mixing, both the MICs in the cells of the WT X WT and KO X WT conjugations moved out from the MAC pockets and elongated (Figure 3.2 A). At 4h after mixing, we can see crescent MICs in both conjugants. At 5h, the cells in the WT X WT control were conjugating normally and four meiotic products could be seen. The KO X WT had no more delay after the formation of crescent MICs. At 6h of mix, WT control was probably undergoing pronuclear mitosis, or even pronuclear exchange. We can see the anlagen I from the WT control at 7h after mixing and anlagen II from 8h post mixing (Figure 3.2 B). Anlagen II can be seen from the 9h time point in the KO X WT. The old Mac in the cells of the WT control began to undergo apoptosis from 9h post mixing, and some were complete after 10h.

The level of pairing efficiency of the wild-type control is very high (93% on average, Table 3.1), and the level of pairing efficiency of an msh2 knockout cell and a wild type cell was reduced (65% on average, Table 3.1).

In order to confirm the completion of conjugation, single pairing cells were isolated at 5-6h mating and were resuspened in the same Tris buffer. The selected pairs completed the conjugation within 14 hours at 30 °C in a moist chamber. The conjugated single cells were spun down and resuspended in SPP media. Following growth, cultures were tested for drug resistance. As the cells are from CU427 and CU428 strains, cyclohexamide, 6-methylpurine and paramomycin resistance were tested. If a conjugation between a CU427 cell and a CU428 cell is complete, the mated cells should be resistant to cyclohexamide (25 μg / mL) and 6-methylpurine (15 μg / mL), and sensitive to paramomycin (200 μg / mL).

The clonal descendants of the wild type pair and of the mixed pair were resistant to cyclohexamide (25 μg / mL) and 6-methylpurine (15 μg / mL), and sensitive to paramomycin (200 μg / mL). Thus, the pairing cells in both the WT control and the KO X WT completed the conjugation.

WT X WT: 3h-6h;



KO*MSH2* X WT: 3h-6h;

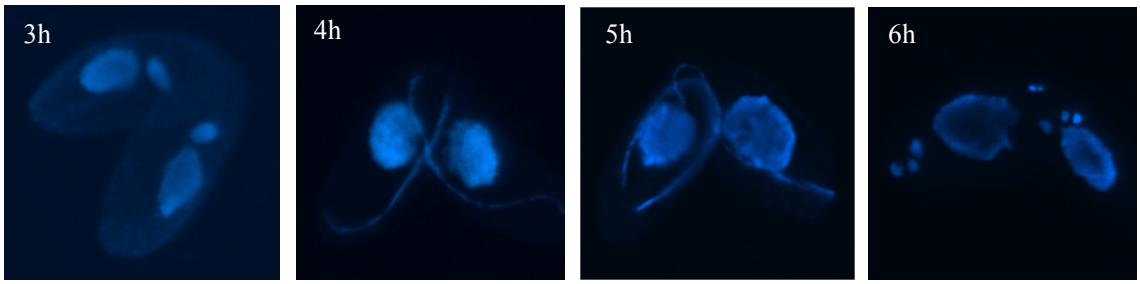
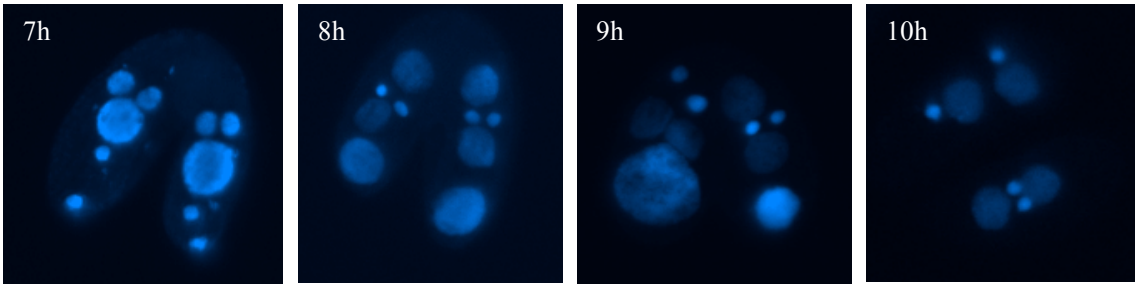


Figure 3.2 A: The conjugation of two wild type cells with different mating types, and a MAC msh2 KO cell and a wild type cell with different mating types, 3h-6h of mix.

WT X WT: 3h: meiotic beginning; 4h: crescent MIC; 5h: meiosis II; 6h: pronuclear differentiation;

KO X WT: 3h: meiotic beginning; 4h: crescent MIC; 5h: chromosome condensation; 6h: pronuclear differentiation;

WT X WT: 7h-10h.



KO*MSH2* X WT: 7h-10h.

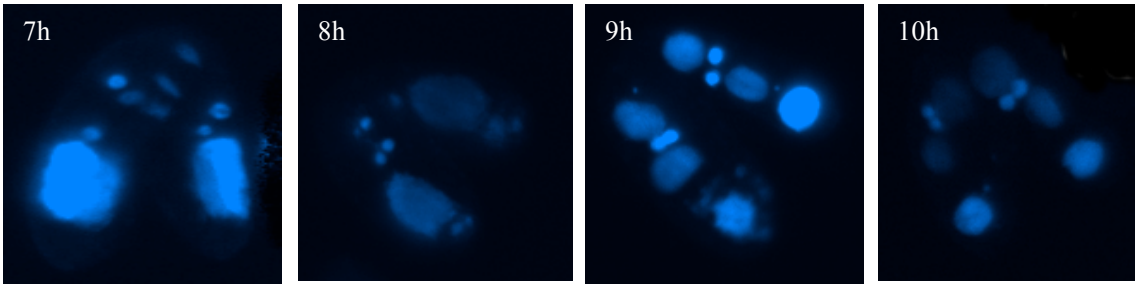


Figure 3.2 B: The conjugation of two wild type cells with different mating types, and a MAC msh2 KO cell and a wild type cell with different mating types, 7h-10h of mix.

WT X WT: 7h: Mac anlagen I; 8h: Mac anlagen II; 9h: Mac apoptosis; 10h: Mac apoptosis complete.

KO X WT: 7h: pre-zygotic mitosis; 8h: prenuclear differentiation and exchange; 9h-10h: Mac anlagen II.

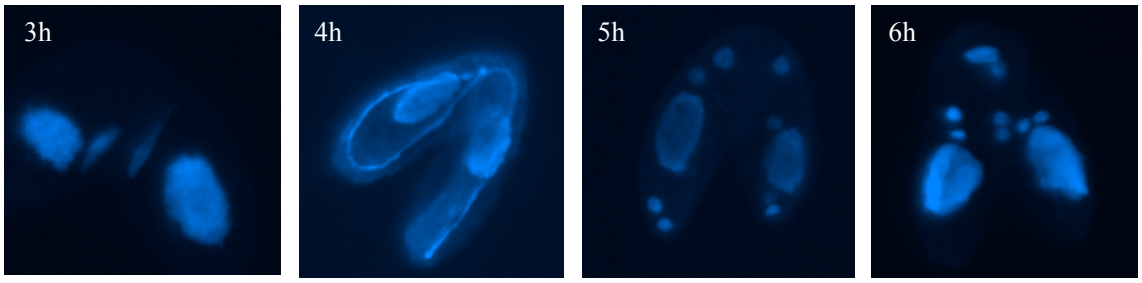
3.1.3 The conjugation of two *Msh2* knockout cells

The conjugation of two msh2 knockout cells with different mating types was set up to study the phenotype of the KO cells. The wild type pair was the control. While the cells of the WT control were conjugating normally at a high level of pairing efficiency, the KO X KO conjugation was totally different. The pairing level between the KO cells was very low (6.3% on average, Table 3.1), compared to the level of the WT control.

Some pairs of the KO cells appeared at 4h after mixing (Figure 3.3 A), and MICs moved out from their Mac pockets and became visible from 5h. However, the conjugation between KO cells stopped after the MICs became visible; no crescent MICs could be seen throughout the experiment. At 10h post mixing, MICs became invisible again and paired cells separated (Figure 3.3 B).

Single pairing cell isolation was also done in KO X KO to check the conjugation. Single pairing cells were isolated at 5-6h post mating and were resuspened in Tris buffer. Once they separated, they were spun down and resuspended in growth medium.

WT X WT: 3h-6h;



KO X KO: 3h-6h;

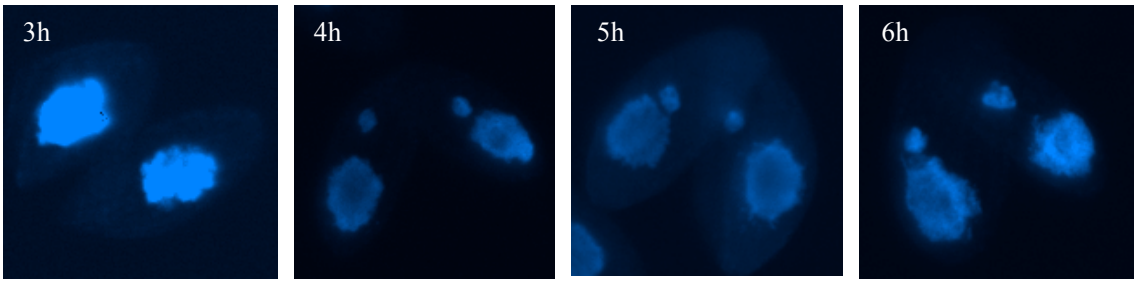
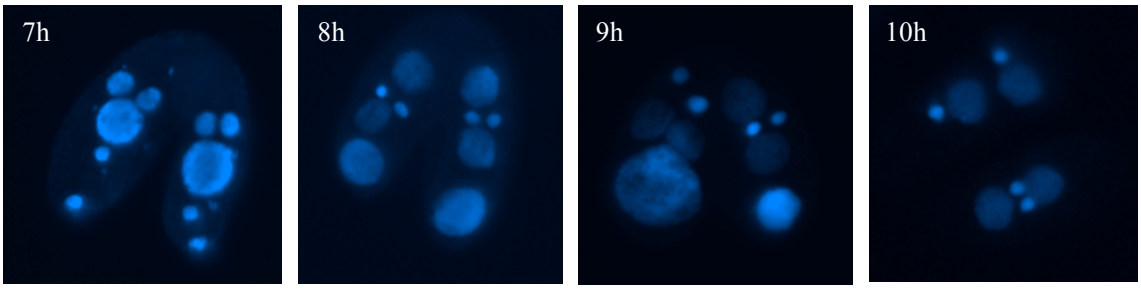


Figure 3.3 A: The conjugation of two wild type cells with different mating types, and two MAC msh2 KO cells with different mating types, 3h-6h of mix.

WT X WT: the same in Figure 3.2, 3h-6h;

KO X KO: 3h: single cell; 4h: MIC move out and cell pairing; 5h-6h: meiosis arrest, no Mic crescent;

WT X WT: 7h-10h.



KO X KO: 7h-10h.

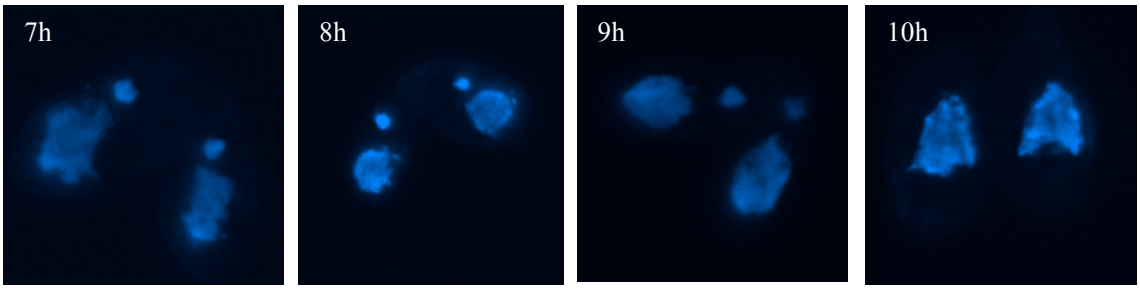


Figure 3.3 B: The conjugation of two wild type cells with different mating types, and two MAC msh2 KO cells with different mating types, 7h-10h of mix.

WT X WT: the same in Figure 3.2, 7h-10h.

KO X KO: 7h-9h, meiosis arrest, no MIC crescent; X: 10h, separated cells.

As the cells are from CU427 and CU428 strains, cyclohexamine, 6-methylpurine and paramomycin resistances were tested. The WT control appeared resistant to cyclohexamide (25 μg / mL) and 6-methylpurine (15 μg / mL), and sensitive to paramomycin (200 μg / mL). The separated KO cells were paramomycin resistant and cyclohexamide and 6-methylpurine sensitive. Thus, the cells of the KO X KO did not complete conjugation.

3.1.4 Cell Pairing Levels of the Conjugations

	WT X WT	KO X WT	KO X KO
1	95%	68%	13.0%
2	93%	63%	16.4%
3	91%	65%	18.0%
Average	93%	65.3%	15.8%

Table 3.1 Levels of cell pairing efficiencies during conjugations of WT and msh2 KO.

The data above were calculated 4h after mixing of the starved cells. Averages were calculated for three replicates for each experiment. These numbers show the different levels of pairing efficiencies of different conjugations. WT X WT is the control to show that the WT cells have normal ability to pair and mate at high frequency after induction. KO cells show weak ability to pair and the ability was enhanced when they met WT cells.

3.1.5 The MICs of the separated *Msh2* knockout cells after pairing

In the KO X KO conjugation, the paired msh2 knockout cells probably separated after 9h of mix. The MICs in those cells became invisible again. These MICs could either have moved back to their Mac pockets, or been degraded after the separation. More conjugations were set up to determine the destiny of the MICs.

If cells have mated, they have to undergo vegetative divisions for about 90 generations to become sexually mature again. In the msh2 knockout conjugation, the cells did not mate. These cells were grown up in rich media and starved over night. The separated msh2 knockout (paired CU427 X CU428) was mixed with a wild type (B2086) that has a third mating type.

Most of the cells did not conjugate. The paired cells (10%, Figure 3.4) have abnormal anlagen II formed from 8h of mix. The MICs in the KO cells were defective after pairing.

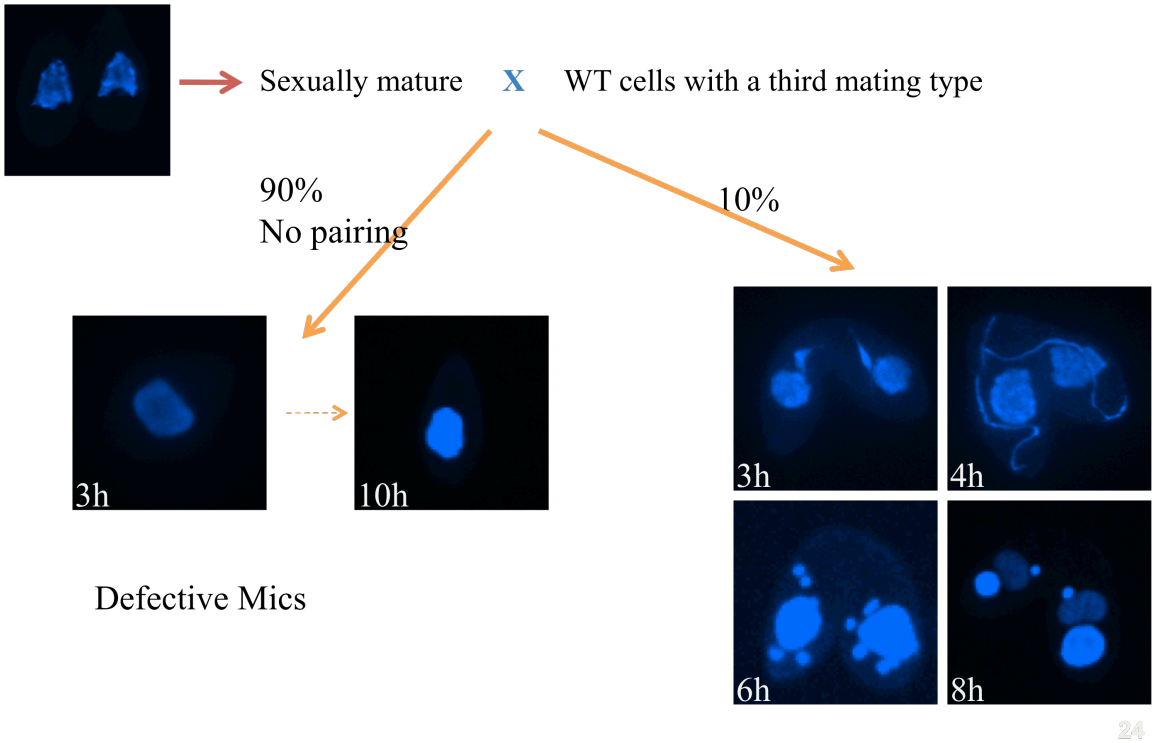


Figure 3.4 Conjugation between the separated msh2 KO cell and the WT cell with a third mating type.

90 % of the cells did not conjugate. 10% of the cells paired but had abnormal anlagen II.

3.1.6 Potential germline aging of the *Msh2* knockouts

The complete msh2 knockouts were maintained at room temperature after confirmation. After about six month, the cells become infertile such that no Mac anlagen could be seen during the conjugation with a wild type. It is probably due to the germline aging (Simon, 1979) induced by the knockout. Germinal aging happens in wild-type cells and mutant cells. In wild-type cells, the aging is only significant after 9-11 years. WT cells are relatively stable for the aging. The manifestations of germinal aging in *T. thermophila* include macronuclear retention during conjugation where the normal replacement of the old macronucleus by a new one fails to occur, and cells die. The aging may be delayed by reducing the number of cell fissions, and can be solved by storing *Tetrahymena* strains in liquid nitrogen.

Potentially aged msh2 knockout cells failed to complete conjugation. The old MAC was retained and no new Mac anlagen were formed; cell conjugation stopped at the stage of pronuclear exchange and fusion. In addition, the mating efficiency of the cell conjugation was greatly reduced.

3.2 Phenotypes of the 2-AP or MMS Treated MSH2 MAC Knockouts in *T. thermophila*.

The MSH2 KO cells could mate with a wild type cell with a different mating type, which indicates that the number of the replication errors occurring during S phase DNA replication of the KO is below the detection threshold of G2/M checkpoint. In order to study the function of Msh2 protein in the S-phase checkpoint, 2-AP (Adenine analog) or MMS (alkylating agent) were used in an attempt to introduce enough errors to initiate the G2/M checkpoints.

3.2.1 Chemical mutagens 2-AP and MMS introduce mutations and are the sources of genotoxic stress

The msh2 Mac knockout strains become sterile after only six months after breeding. It is probably due to the increased amount of mutations accumulated during DNA replication without correct repair in the nuclei. Certain amount of mismatches can cause DNA damage, like the damage of replication fork, which results in cell cycle arrest or apoptosis.

In order to study the role of the Msh2 protein in the intra-S phase checkpoint response in the diploid micronucleus, 2-AP and MMS mutagens were used to induce mismatches and bring stress to the cells, which triggers the activation of the damage sensor / transducer proteins ATR. ATR protein kinase phosphorylates the effector kinase Chk1p (in mammals), Rad53p (in budding yeast), or Cds1p (in fission yeast), and other regulatory proteins (see Introduction).

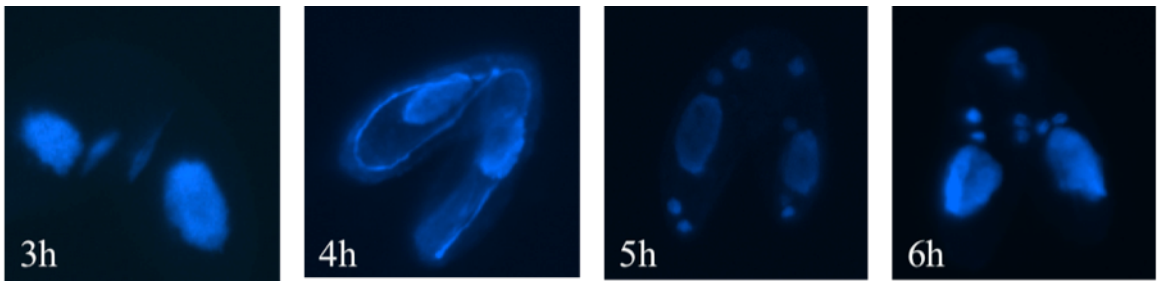
3.2.2 The conjugation of 2-AP or MMS treated wild type cells with different mating types

The non-treated WT X WT control has a high level of pairing efficiency (~93%). The level of pairing efficiency of the conjugation between two 2-AP or MMS treated wild type cells, with different mating types, is also high (~92% and ~96%), the same level of efficiency as the conjugation between two non-treated wild type cells (Figure 3.5). Most of the treated WT cells completed the conjugation and significant Mac anlagen can be seen after 7h of mixing (figure 3.5 B).

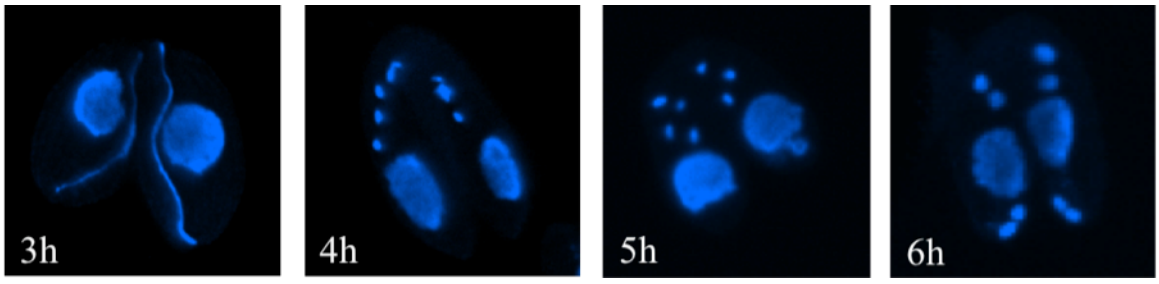
3.2.3 The conjugation of a 2-AP or MMS treated *Msh2* knockout cell and a non-treated wild type cell

The non-treated KO X WT is the control, which has a level of pairing efficiency at 65%. The level of pairing efficiency of the conjugation between 2-AP or MMS treated KO cells and non-treated wild type cells, with different mating types, is low (~21% and ~4%), in which about 80-90% about the cells could not pair, and could not enter meiosis consequently (Figure 3.6).

WT X WT non-treated, 3h -6h.



WT X WT 2-AP treated, 3h -6h.



WT X WT MMS treated, 3h -6h.

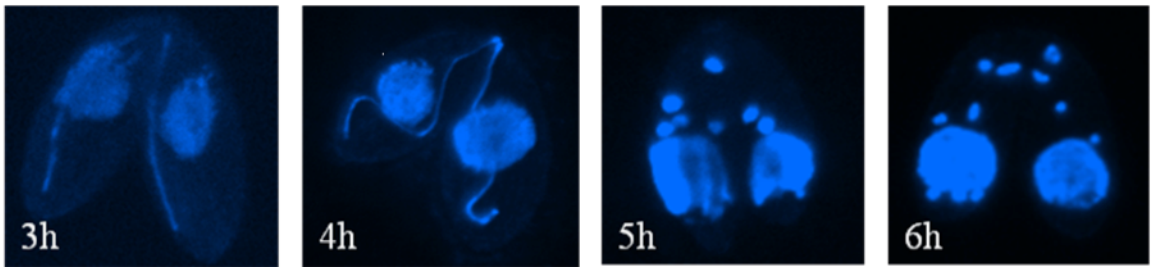
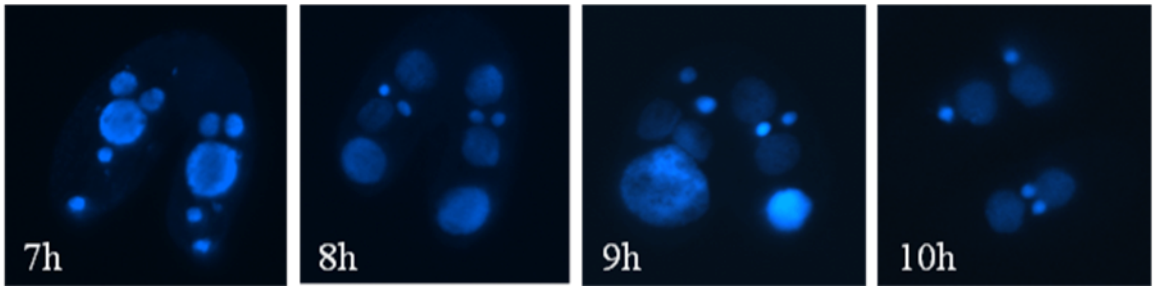
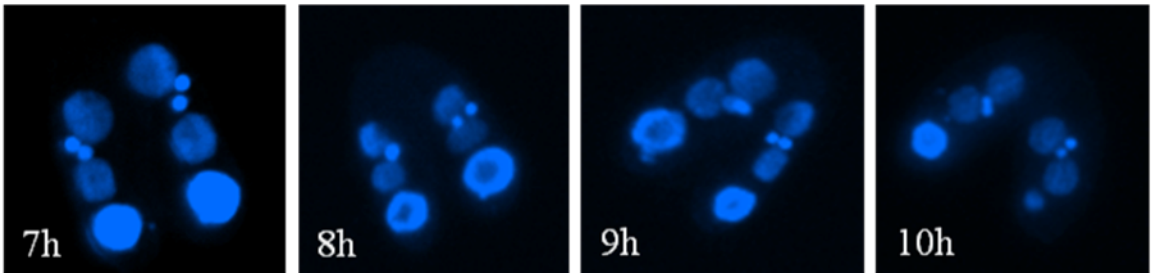


Figure 3.5 A: The conjugation of two wild type cells with different mating types 3h-6h, that were non-treated, 2-AP treated, and MMS treated.

WT X WT non-treated, 7h -10h.



WT X WT 2-AP treated, 7h -10h.



WT X WT MMS treated, 7h -10h.

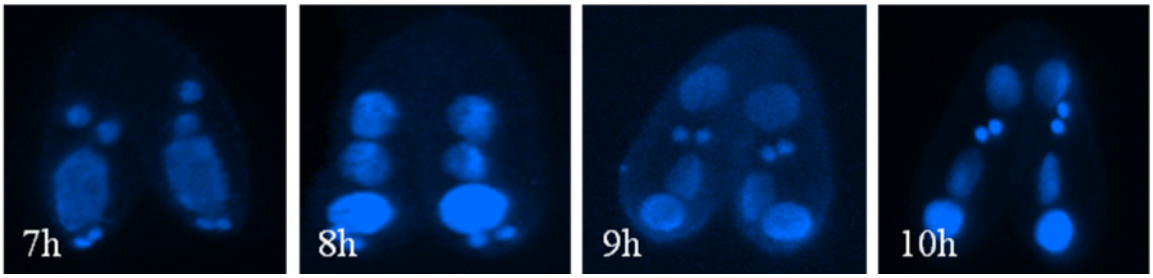
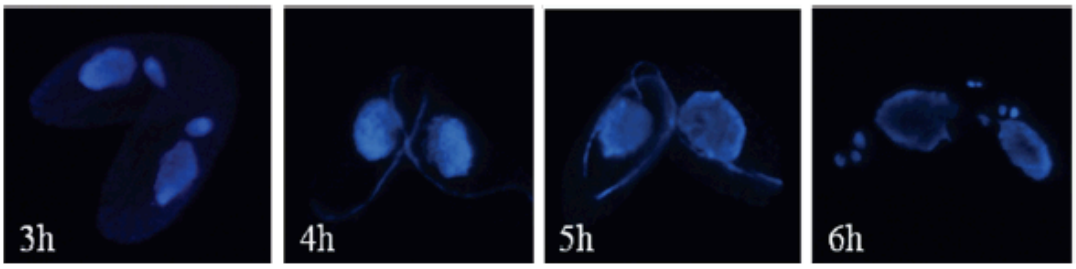
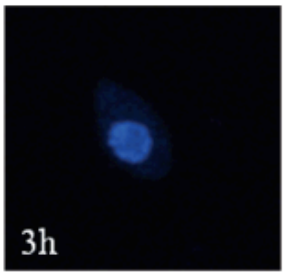


Figure 3.5 B: The conjugation of two wild type cells with different mating types 7h-10h, that were non-treated, 2-AP treated and MMS treated.

Non-treated KO *msh2* X WT, 3h -6h.

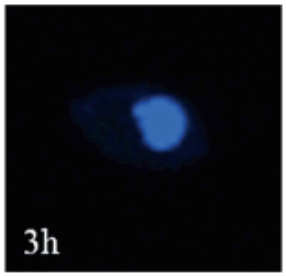


2-AP treated KO *msh2* X WT, 3h -6h.



~79% cells had **no** conjugation

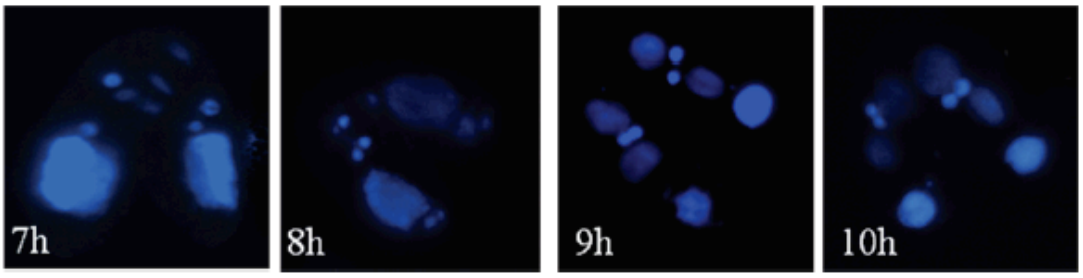
MMS treated KO *msh2* X WT, 3h -6h.



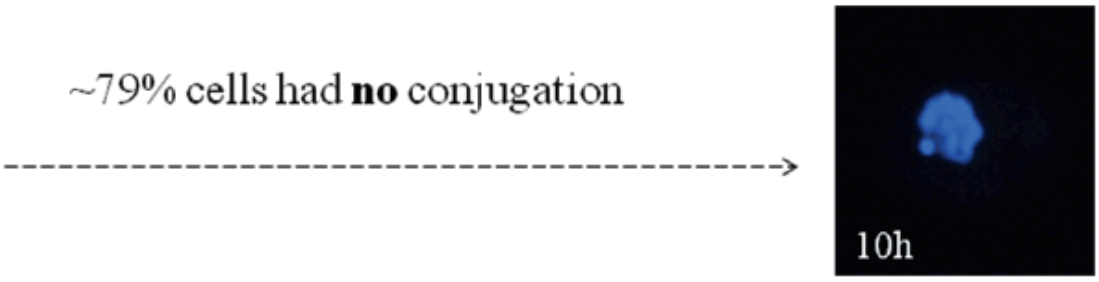
~97% cells had **no** conjugation

Figure 3.6 A: The conjugation between an *msh2* knockout cell non-treated, 2-AP treated, and MMS treated, and a non-treated wild type cell, 3h-6h.

Non-treated KO *msh2* X WT, 7h -10h.



2-AP treated KO *msh2* X WT, 7h -10h.



MMS treated KO *msh2* X WT, 7h -10h.

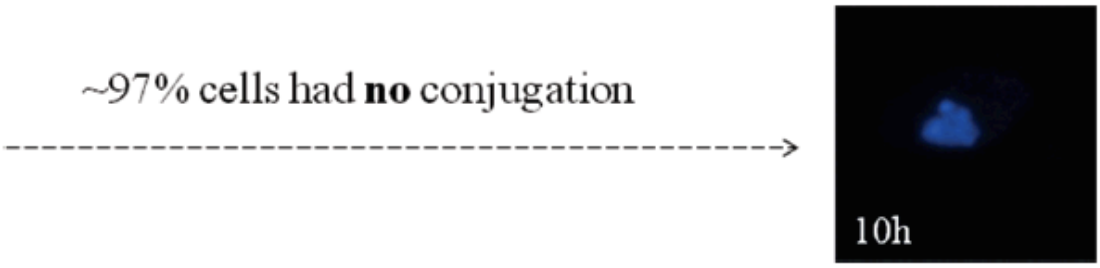


Figure 3.6 B: The conjugation between an *msh2* knockout cell non-treated, 2-AP treated, and MMS treated, and a non-treated wild type cell, 7h-10h.

Calculation of the pairing efficiency:

	WT X WT	KO msh2 X non-treated WT
Non-treated	93%	65%
2-AP treated	92%	21%
MMS treated	96%	4%

Table 3.2 Levels of the pairing efficiencies of the conjugations of non-treated WT and msh2 KO.

2-AP or MMS treated WT X WT has the same level of pairing efficiency with non-treated WT X WT. Although non-treated KO X WT has a lower pairing level than WT X WT, two thirds of the KO X WT could not conjugate after the 2-AP treatment to the KO cells. MMS seems to have a stronger effect to the KO X WT conjugation than that of 2-AP. Almost all MMS treated KO cells could not pair with non-treated WT cells.

3.3 The msh2 MIC Knockouts in *T. thermophila*.

In contrast to macronuclear gene knockout, micronuclear gene knockout means the gene in both the macronucleus and the micronucleus were knocked out. There is no Msh2 gene in either micronuclei or macronuclei of *T. thermophila*.

MIC KO msh2 have been done and verified by genomic PCR and RT-PCR (Figure 3.7). In addition, the MIC KO was crossed with WT to check the KO in the Mic. All of the progeny of the cross were paramomycin-resistant. Thus, both alleles are deleted in the Mic.

As mentioned in the experimental section, during the MIC KO transformation, each transformed pair produces four karyonidal clones which are genetically identical but can have different mating types. Single cells were isolated and grown up, and the mating types of them were tested by mating with other different mating types.

Similar to the macronuclear msh2 KO cells conjugation, micronuclear msh2 KO cells also can mate with wild type cells normally. The mating efficiency of the MIC msh2 KO pair is much lower than the MAC msh2 KO pair. Single cell isolation is hard to implement. Drug tests have been done to the conjugated mixed pair. The progeny were pm-resistant, cy-resistant and 6mp-resistant. For the KO X KO, almost all of the MIC KO cells between two different mating types could not pair.

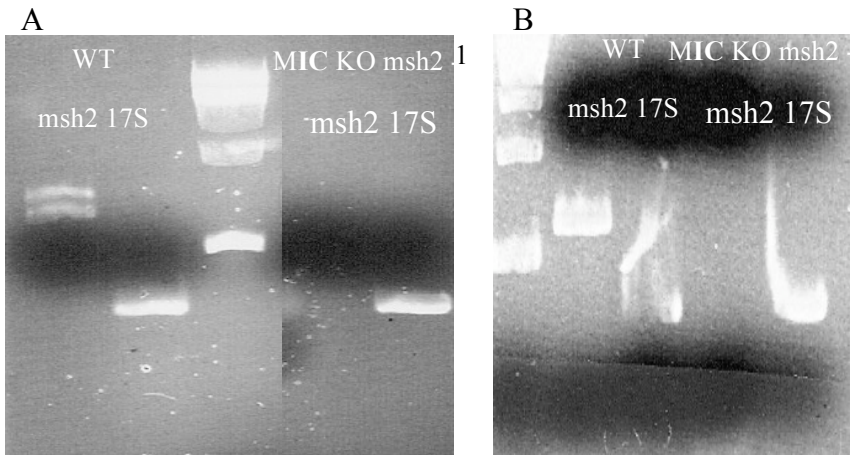


Figure 3.7: mRNA RT-PCR of the MIC *msh2* knockouts.

A: *msh2*MIC KO-1; B: *msh2* MIC KO-2.

3.4 Phenotypes of the Mlh1 MAC Knockouts in *T. thermophila*.

3.4.1 The *Mlh1* MAC knockouts

The gene of Mlh1 homolog has been completely knocked out in the macronuclei of two strains of different mating types in *T. thermophila*, CU428 and B2086. The complete mlh1 knockouts were confirmed by Genomic PCR and mRNA Reverse Transcriptase PCR (the same as the KOmsh2). Knockout cells were grown up to $5x10^5$ cells / ml before their genomic DNA and messenger RNA were isolated. For the RT-PCR, in the KO cells, there are products from the reactions of the 17S rRNA primers but no product from the reactions of the mlh1 primers; in the WT control, there are products from both of the reactions of the 17S rRNA primers and the mlh1 primers (the same as Figure3.1).

3.4.2 The conjugations of the Mlh1 KO X WT and the Mlh1 KO X KO

Both the conjugation of KO X WT and KO X KO were set up and compared with WT X WT control. Cells were grown up, starved and mixed in equal numbers (described before).

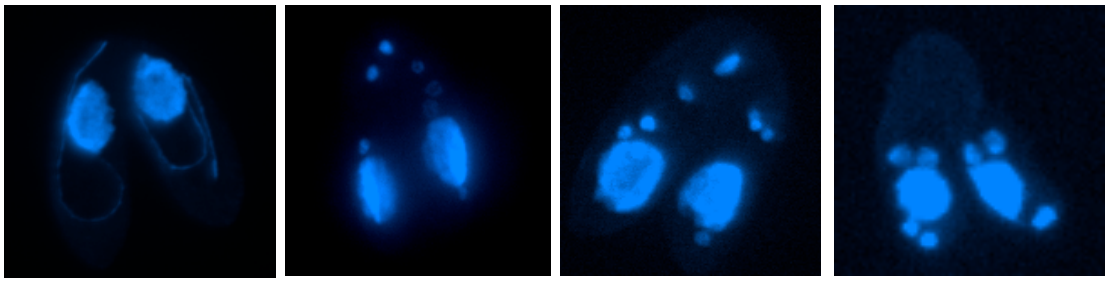
In this WT X WT control, cells conjugated normally at high level of pairing efficiency (~99%). The conjugation of the cells in the KO X WT was delayed about 1 hour compared to the WT control, with reduced level of pairing efficiency (~45%). At 3h post mixing, the cells in the WT control formed the crescent MICs; the cells in the KO X WT began to elongate; the cells in the KO X KO still had their MICs in the Mac pockets. The cells in the WT control had the anlagen II formed at 7h of mix, the KO X WT at 8h, and the KO X KO at 9h. Therefore, the conjugation of the cells in the KO X KO was about 1 hour later than that in the KO X WT with severely reduced level of pairing efficiency (~13%). Totally it was 2 hours later of the KO X KO conjugation than the WT control. However, the paired cells in both KO X WT and KO X KO could complete the conjugation.

Single pairing cell isolation was also done in WT X WT, KO XWT and KO X KO to verify the conjugations. Single pairing cells were isolated at 5-6h of mating and

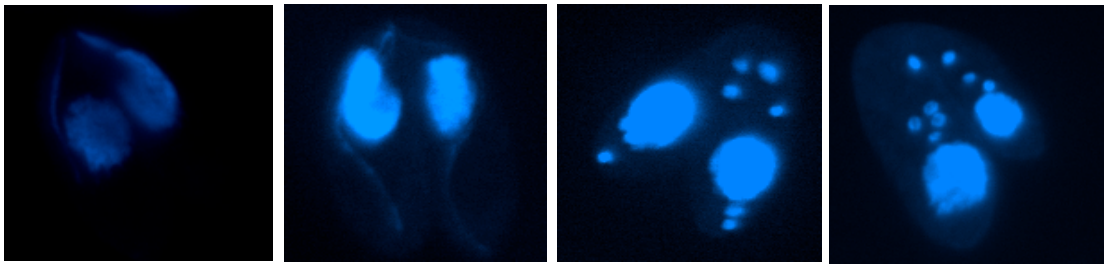
were resuspended in Tris buffer. Once they separated, they were spun down and resuspended in growth medium.

As the cells are from CU428 and B2086 strains, 6-methylpurine and paramomycin resistances were tested. All of the cells isolated from the WT control, KO X WT and KO X KO appeared resistant to 6-methylpurine (15 μg / mL), and sensitive to paramomycin (200 μg / mL). Thus, all of the paired cells of the three combinations completed the conjugation.

WT X WT, 3h-6h



KO*MLH1* X WT, 3h-6h



Mlh1 KO X KO, 3h-6h

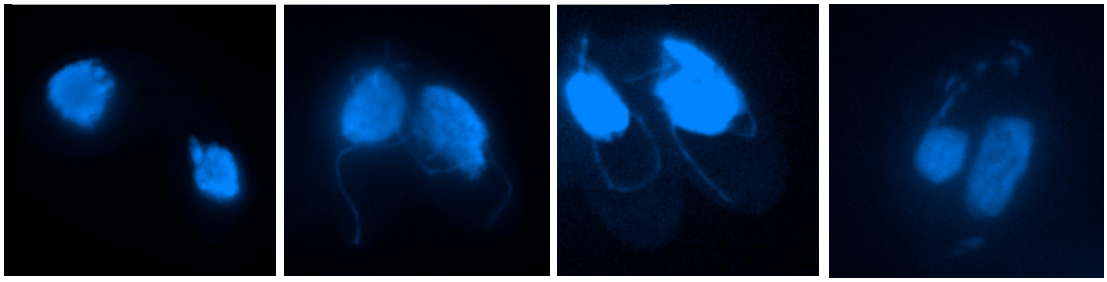


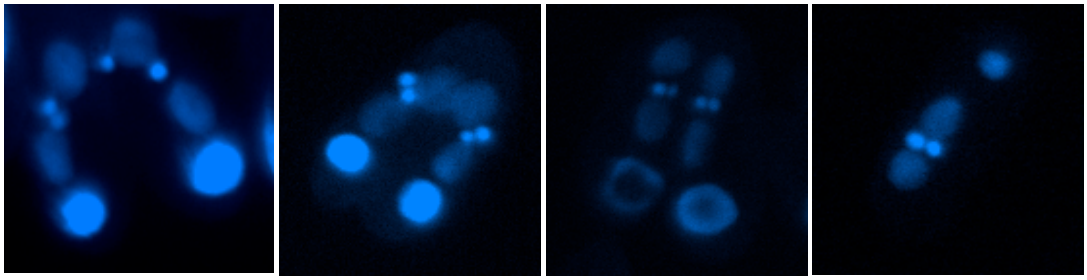
Figure 3.8 A: The cell conjugation of WT X WT, mlh1 KO X WT, mlh1 KO X KO, 3h-6h.

WT X WT: 3h: crescent MIC; 4h: meiosis II; 5h: pronuclear differentiation; 6h: 2nd post-zygotic mitosis, Mac anlagen I;

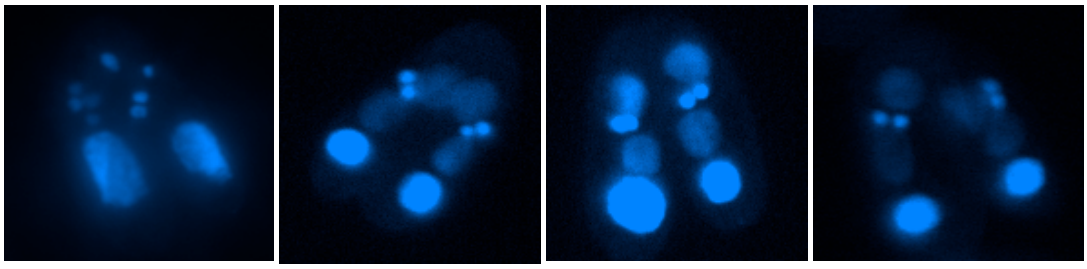
Mlh1 KO X WT: 3h: meiotic beginning; 4h: crescent MIC; 5h: meiosis II; 6h: pronuclear differentiation;

Mlh1 KO X KO: 3h: meiotic beginning; 4-5h: crescent MIC; 6h: meiosis I;

WT X WT, 7h-10h



KO*MLH1* X WT, 7h-10h



Mlh1 KO X KO, 7h-10h

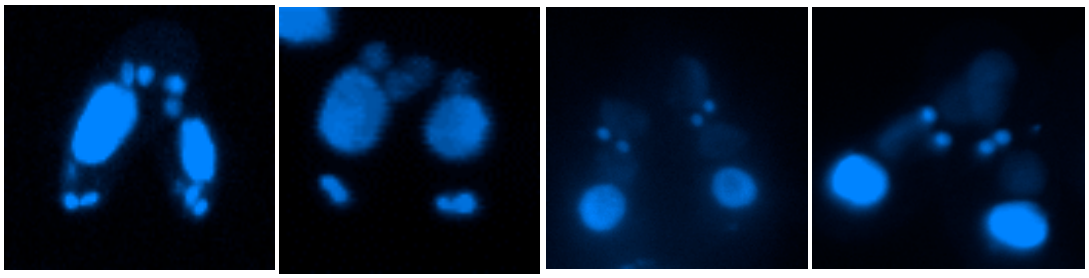


Figure 3.8 B: The cell conjugation of WT X WT, mlh1 KO X WT, mlh1 KO X KO, 7h-10h.

WT X WT: 7-8h: Mac anlagen II; 9h: Mac apoptosis; 10h: pair separation.

Mlh1 KO X WT: 7h, pronuclear exchange and fusion; 8-10h: Mac anlagen II.

Mlh1 KO X KO: 7h, meiosis II; 8h: 2nd post-zygotic mitosis; 9-10h: Mac anlagen II.

4 DISCUSSION

The purpose of this project is to study the function of MMR protein homologues MSH2 and MLH1 during meiosis in *Tetrahymena thermophila*. *T. thermophila* is a complicated microorganism. Its double nuclei are believed to have clearly non-overlapping functions. It is not known, however, whether these proteins have a role in both these nuclei. I have used MSH2 knockout cells to study the effect of these proteins in different aspects of the cell cycle

4.1MSH2 Is Indispensible for the Formation of Crescent Micronuclei

The paired msh2 knockout cells show their willingness to initiate meiosis. Their MICs moved out from the Mac pockets post 4h of cell mixing. However, no crescent MIC has been formed throughout the conjugation process, and then the MICs become invisible again at 10h of mixing. When neither of the mixed partners has MSH2, the MIC cannot elongate and subsequent events are stopped. It is proposed that the elongated shape of meiotic MIC promotes the parallel arrangement of chromosomes and supports the juxtaposition of homologous

regions in the absence of a SC. In yeast, MSH4 and MSH5 are restricted to meiosis and have played a role in recombination during resolution of crossovers and the recognition of recombination intermediates. There is no homologue of MSH4 or MSH5 in *T. thermophila*. Thus, it is suggested that MSH2 may have the function in recombination instead of MSH4/MSH5.

The conjugation of a wild-type cell and a MSH2 knockout cell is one hour later than the wild-type pair. It is possible that at the beginning of the conjugation of the mixed pair, only the WT partner has the MSH2 protein; and the protein is transported to the KO partner. Thus, two cells share the protein from only one cell, which halves the amount of the MSH2 in the WT cells (Figure 4.1). Reduced amount in one cell may trigger the protein synthesis. The transportation of MSH2 may also need time. Both the synthesis and transportation may explain the delay. When the amounts of MSH2 in both cells reach the threshold, the crescent can be formed. Although there is a delay, the mixed pair still forms the crescent MIC and completes the conjugation.

The data from MSH2 gene expression assay (Figure 4.2, Annandale, submitted) shows that the expression of MSH2 reaches the highest level from 3h to 5h, which

is the period of crescent MIC formation. This information supports the hypothesis that MSH2 is required for the formation of crescent MIC.

A recently published paper (Loidl, 2009) explained a possibility of the crescent MIC arrest. DNA double-strand breaks (DSBs) are required for entering the crescent stage in *Tetrahymena*. DSBs can be caused by spo11 or DNA-damaging agents. MIC elongation following Spo11p-induced DSBs or artificially induced DNA lesions is probably a DNA-damage response. During meiosis, DSBs are formed by SPO11 protein and meiotic DSBs trigger phosphokinase transduction pathway, which is suppressed by defects of ATM/ATR kinase or ATR orthologue. MIC elongation does not occur in the absence of Spo11p-induced DNA DSBs. Another paper (Wang, 2003) shows that MSH2 protein interacts with ATR kinase to form a signaling module and regulate two branches of DNA damage responses to DNA methylation caused by MNNG. It is probable that the MIC elongation during the meiosis of the two msh2 knockout strains with different mating types was inhibited because there was no ATR-MSH2 signaling module or no DNA damage sensor to activate ATR to mediate the phosphokinase transduction pathway for the MIC elongation.

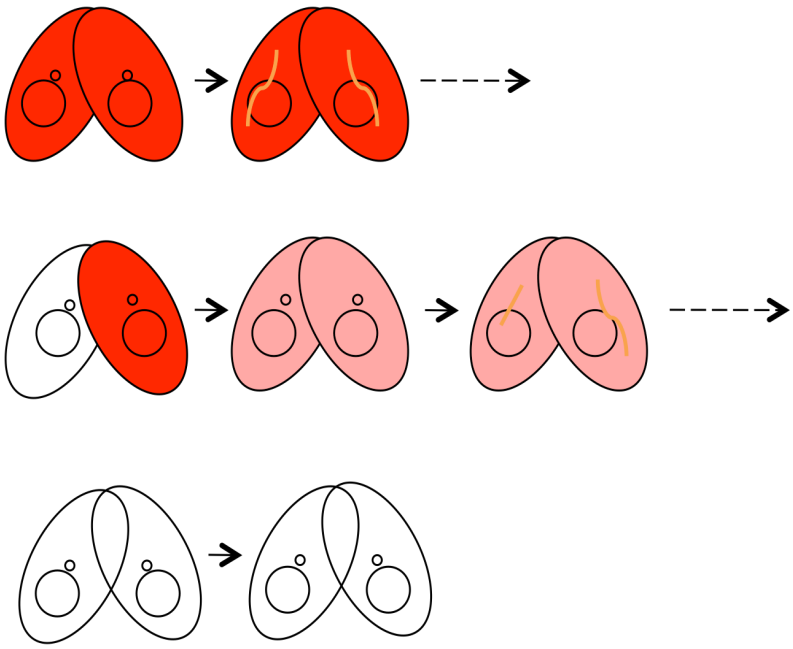


Figure 4.1: The theory of the formation of the crescent MIC.

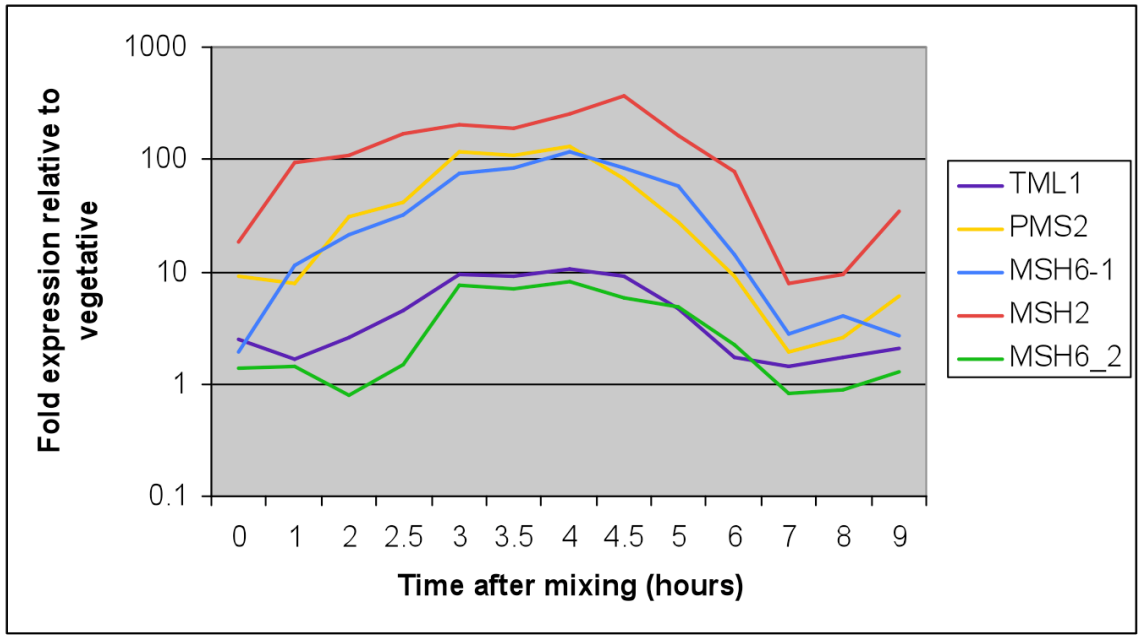


Figure 4.2: Expression of MMR genes in conjugating cells relative to vegetative cells (Annandale, submitted). The red line shows the msh2 gene expression. This expression reached its highest level during the conjugating process at 4-5 hour after mixing, which is the period that crescent micronucleus is formed.

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4.2 Msh2 Protein May Be Involved In the Meiotic Intra-S phase Checkpoint

The amount of 2-AP or MMS used has no effect on the mating efficiency of a treated WT strain with a non-treated WT strain; the conjugation efficiency is similar to that of the non-treated WT X WT. It is probable that the treated WT cells are able to repair the mismatches by the MMR proteins. Therefore, meiosis is not affected.

When Msh2 mutant cells, previously treated with 2-AP or MMS, are induced to conjugate with non-treated WT, the level of pairing efficiency is severely reduced, and most of the cells cannot pair at all. This indicates that, in this case of MSH2 mutant, G2/Meiosis checkpoint arrests the meiotic cell cycle and most cells cannot pass the G2/Meiosis transition; the intra-S checkpoint is no longer functional. It is probable that treated cells cannot repair the mismatches; when the amount of the mismatches is above the threshold of the detection level, the meiosis would be arrested by the checkpoint.

These findings indicate that MSH2 is not a component of the G2/Meiosis checkpoint. It is likely that MSH2 has a role in premeiotic intra-S phase checkpoint.

From Vincent O'Brien's review paper (O'Brien, 2006), MSH2 and MLH1 are shown to be required for the G2/Mitosis checkpoint signaling. MMR-deficient cells exhibit defects in G2/Mitosis cell cycle arrest and cell killing when treated with DNA damaging agents. However, the definition of S phase checkpoint is not mentioned in O'Brien's paper. From Kevin D. Brown's paper (Brown, 2003), MSH2 and MLH1 are shown to be required for S-phase checkpoint activation. In vitro and in vivo approaches both show that MSH2 binds to CHK2 and that MLH1 associated with ATM. These findings indicate that the mismatch repair complex formed at the sites of DNA damage facilitates the phosphorylation of CHK2 by ATM. Thus, MSH2 and MLH1 are required for both the G2/Mitosis and S phase checkpoints.

From my results, MSH2 is not a component of G2/Meiosis checkpoint. If MSH2 is required for the premeiotic S phase checkpoint, the drug treated MSH2 mutant cells with certain mismatches can pass the S checkpoint but will be arrested at G2/Meiosis; if MSH2 is not necessary for the premeiotic S phase checkpoint, the

mutant cells will be arrested at S phase and the mismatches will be sensed and activate the S phase checkpoint. However, whether the mutant cells stay at S phase or G2 phase is not clear. In order to determine the stage that the cell arrested at, I can measure the amount DNA content by flow cytometry (Ferullo, 2009), using a DNA-specific dye such as PicoGreen. The dye can be detected by most commercial flow cytometers. The nucleotide ethynyl-deoxyuridine (EdU) labeled fluorescent dye incorporation can also be used to measure and visualize newly replicated DNA in fixed cells under non-denaturing conditions. The DNA synthesis process in *T. thermophila* can be determined following this method, which helps to understand the events happened during meiotic S phase of the drug treated KO cells.

Since 2-AP or MMS are mutagens, they may cause toxicity to cells. However, at vegetative life state, KO (MMR-deficient) cells showed increased resistance to the drugs compared to WT cells (O'Brien, 2006). Thus, toxicity seems to be a neglectable reason to explain the inability of the cells to enter meiosis.

4.3 Whether MSH2 and MLH1 Function In the Same MMR Pathway Remain To Be Confirmed

The phenotypes of MSH2 KO X KO and MLH1 KO X KO are different. Although the pairing efficiencies in both conjugations are very low, the paired cells in MSH2 KO X KO cannot form crescent micronuclei and therefore cannot complete the conjugation while the paired cells in MLH1 KO X KO can complete the conjugation. It is suggested that in human both the MSH2 and the MLH1 homologues functions in the same MMR complex because there is only one nucleus in human cells. However, the localization of the MSH2 protein is probably limited in the MIC while the localization of the MLH1 protein is probably limited in the MAC during conjugation in *T. thermophila* (Figure 4.3 Annandale, submitted). MSH2 and MLH1 can hardly work in the same MMR complex or pathway. The localization of the PMS2 protein is probably limited in the MIC, thus the MLH1 and PMS2 homologues in *T. thermophila* cannot form a heterodimer to function either. It is hypothesized that the PMS2 homolog functions as a homodimer instead of a heterodimer with the MLH1 in the MMR pathway.

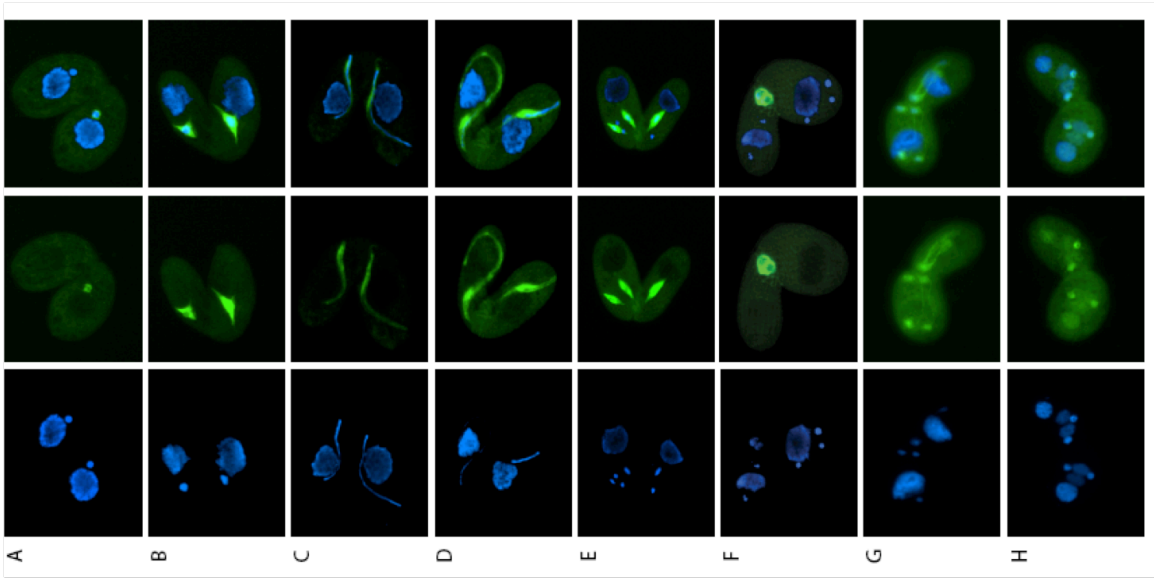

Figure 4.3: PMS2 protein localization (Annandale, submitted). The blue color shows the nuclei and the green color shows the protein. The Pms2 homologue localizes solely to the MIC throughout the conjugation.

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However, since these GFP fused protein have never been shown to be still functional, that is so far no rescue experiments have been done to show that the extra GFP tag has not affected the function of the protein, the localization of the GFP fused protein may not represent the endogenous localization. Therefore further experiments of using 2-AP and MMR to treat MLH1 knockout and MSH2MLH1 double knockouts are necessary to verify this possibility.

In addition, the phenotype of KO X WT of MSH2 KO and MLH1 KO are similar; they both have a 1h delay during the conjugation. It is possible that these two proteins function (not the MMR functions) on a same component or complex, although the two proteins are localized on different compartments; the short of either protein could trigger similar signaling events and lead to the delay of crescent formation during conjugation. Therefore similar to the GFP fused Msh2 experiment proposed before, GFP fused Mlh1 protein can be expressed to rescue the knockout phenotype and observe the intercellular protein transportation between KO and WT cells.

4.4 MSH2 And MLH1 Affect the Cell Pairing of *T. thermophila*

The pairing efficiency of the conjugation between wild-type and MSH2 knockout cells is decreased compared with the wild-type pair; and the decrease is worse in the MSH2 knockout pair. I want to examine the cytoskeleton behaviors during the mating process to further investigate the phenotype of MSH2 mutant cells.

One of the distinct locations of actin is in oral apparatus (OA), and OA is the place where two pairing cells physically connect. One possible scenario is that MSH2 protein may have direct or indirect interaction with actin, which is a major component of cytoskeleton in eukaryotes. MSH2 protein may have a role in controlling actin polymerization within cells to regulate different cellular processes. My results suggest that the msh2 knockout affects the pairing before the cells enter meiosis. Because *Tetrahymena* actin does not bind to phalloidin or NBD-Ph at all (Barak, 1981), it is not possible to stain the cytoskeletal actin in the cell directly. However, the actin cytoskeleton behavior in *Tetrahymena* cells can be studied by transforming them with plasmids containing epitope-tagged *Tetrahymena* actin genes.

Another possibility causing the decrease of the pairing efficiency of KO X KO is that the cells without the MSH2 protein or with unrepaired mismatches may not be a good partner to pair and mate. WT cells may be able to see the disfigured ones when they are mixed together to co-stimulate for the pairing.

In summary, in *Tetrahymena thermophila*, the MMR protein homologues MSH2 and MLH1 may function differently than the homologues in human or yeast. In human and yeast cells, MSH2 and MSH6 proteins function as a heterodimer, similar to the MLH1 and PMS2 proteins. In *T. thermophila*, the localizations of MSH2 and MSH6 proteins are not the same; MSH2 localizes in the MIC and MSH6 localizes in the cytoplasm area. The localizations of MLH1 and PMS2 proteins are also different; MLH1 localizes in the MAC and PMS2 localizes in the MIC (as communicated by Erin Annadale). It is therefore probable that MSH2 and MSH6, or MLH1 and PMS2 proteins can not form dimers to function and each of the proteins may form homodimers. In addition, msh2 and mlh1 knockout strains have different phenotypes during cell conjugation; therefore, these two proteins may not function in the same pathway.

For the MSH2 protein, it is indispensable for the formation of a crescent MIC. It has a significant role in meiosis. However, it showed no function in the meiotic checkpoint. It probably has a role in the intra-S phase or the mitotic checkpoint, because msh2 knockout strains can grow normally even when a certain level of damage is accumulated to activate the checkpoint. *T. thermophila* is a good model to study the roles of these homologues during meiosis. However, the mismatch repair ability of the MSH2 protein still needs to be determined.

5 FUTURE DIRECTIONS

From the data in this thesis, some outstanding questions need to be demonstrated in the future. The indispensability of the MSH2 protein for the crescent MIC formation during meiosis in *T. thermophila* has not been confirmed. In order to illustrate that, the strategy is to clone the construction of plasmid containing GFP-tagged msh2 gene and an inducible MTT promoter, transform the plasmid into the msh2 KO strains, and induce the expression of the MSH2 protein. This rescue experiment is expected to show the recovery of the crescent MIC formation and meiosis I. It can be done in two KO strains with different mating types to study the indispensability of the MSH2 protein (Figure 5.1 I), or done in one KO strain (figure 5.1 II), subsequently crossing this GFP-transformed strain with another KO strain with a different mating type. The intercellular protein transportation can be studied by this method.

In addition, the microtubule cytoskeleton behavior during crescent MIC formation can also be examined to study the potential relation between the MSH2 protein and the microtubule and the actin in meiosis. Both actin and microtubule play a crucial role during cell division (Etienne-Manneville, 2004). MSH2 protein interacts with the ATR (ATM- and Rad3-related) kinase to form a signaling module and regulate the phosphorylation of CHK1 and SMC1 (structure maintenance chromosome 1) in the S phase checkpoint system (Wang, 2003), which may potentially trigger a signal pathway which eventually affects cytoskeleton behavior. Therefore, a plasmid containing epitope-tagged microtubule or actin gene can be constructed and transformed into GFP-tagged $MSH2^{+}$ and $MSH2^{-}$ strains, to study cytoskeleton behaviors of microtubule and actin during crescent MIC formation in *T. thermophila*.

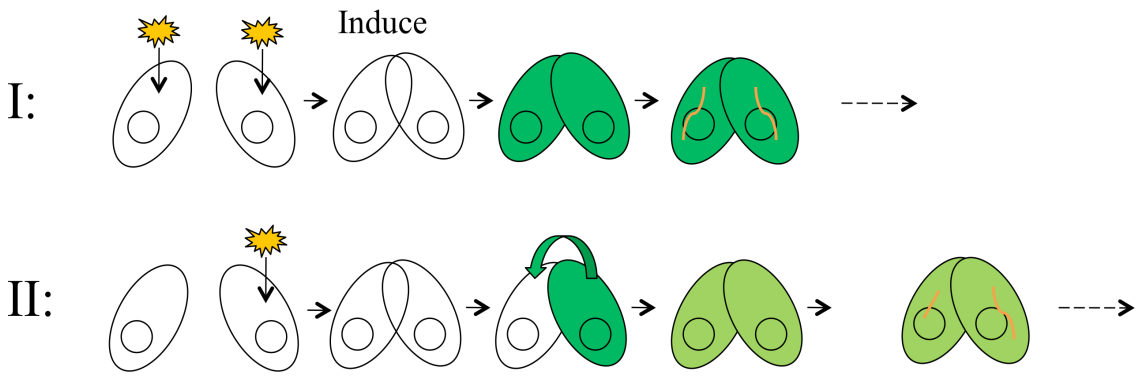

Figure 5.1 Rescue experiments of the MAC msh2 KOs.

I: bombardment to two KO cells of different mating types.

II: bombardment to one KO cell to study the intercellular Msh2 protein transportation.

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7 APPENDIX

The *cds1* MAC Knockouts in *T. thermophila*

The core protein in the intra-S phase checkpoint may be Cds1 in *T. thermophila* (the homolog of Cds1p in fission). In order to determine the role of the Msh2 protein in the meiotic intra-S phase checkpoint, I knocked out the Cds1 gene in two strains of different mating types in *T. thermophila*, CU428 and B2086. I want to test their phenotypes under the 2-AP or MMS treatment.

The cds1 knockout plasmid has been constructed. The plasmid contains a second selective marker rpl29 and the MTT promoter. The knockouts of CDS1 (a major kinase in checkpoint system, Chk2 in human) in *T. thermophila* have been done in the same way as the knockouts of MMR proteins. The vector used to make *CDS1* MAC knockouts was made in this project based on vector pMrpL29B (from Dr. Gorovsky's lab). Different selectable marker was used in this knockout.

The 5' and 3' flanks were cloned on the two sides of the *rpL29* cassette in vector pMrpL29B which gives cycloheximide ressitance. The successful clones of pKOCDS1 were determined by restriction analysis. *Xba*I and *Kpn*I cut the pKOCDS1 to two fragments of 3 kb and 3.9 kb; *Xho*I and *Kpn*I cut the pKOCDS1

to two fragments of 1.1 kb and 5.8 kb (Figure 7.1). Unlike the *neo* cassette, the *rpL29* cassette has an MTT promoter that is inducible by Cd^{2+}. After the biolistic shoot, the cells were put in 1x SPP medium with 1µg / mL cadmium chloride and shaken gently at room temperature over-night. On the next day cycloheximide was added to15 µg / mL starting concentration and the cells were aliquotted to microtitre plates and put at 30°C in a moist chamber. The concentration of cycloheximide has been pushed to 200 µg / mL so far. The efficiency of the knockouts was assessed by genomic PCR and reverse transcriptase PCR of messenger RNA, which is described in 2.2.4. Complete KOs are still in selection.

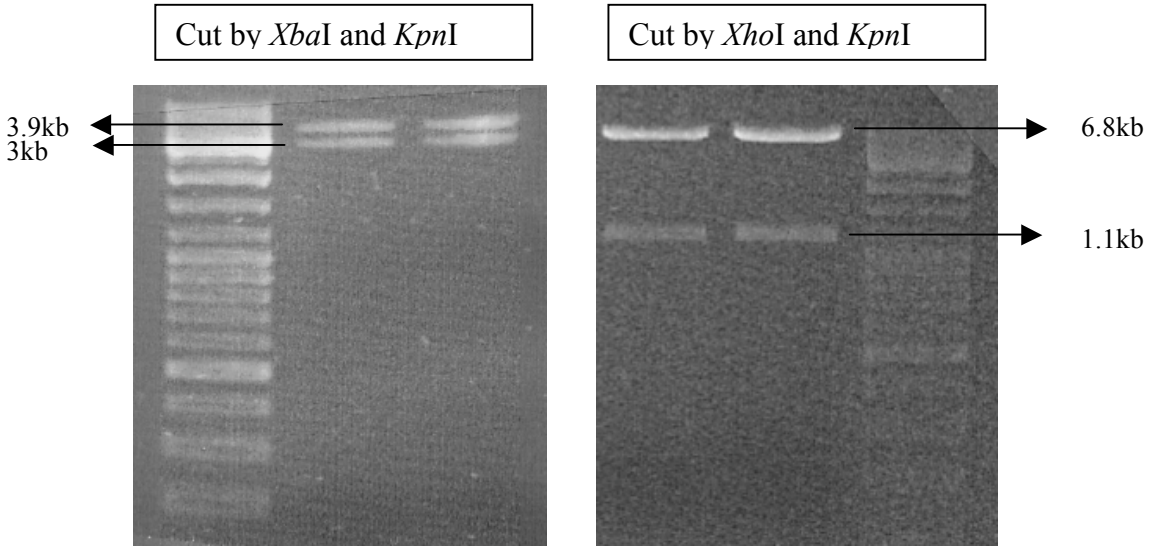

Figure 7.1: Restriction analysis of pKOCDS1.