# Evaluating Transmission Barriers to *Escherichia coli x Saccharomyces cerevisiae* interkingdom conjugation.

Nicholas David Haslett

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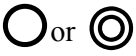
# Abbreviations and Symbols used

| | |
|---|---|
| L | Litre |
| mL | Millilitre |
| µL | Microlitre |
| g | Gram |
| mg | Milligram ($10^{-3}$g) |
| µg | Microgram |
| DNA | Deoxyribonucleic acid |
| $^{o}$C | Degrees Celcius |
| rpm | Revolutions per minute |
| LB | Luria-Bertani medium |
| YPD | Yeast Peptone Dextrose |
| SC | Synthetic Complete |
| mol | Mole |
| mM | millimolar |
| bp | DNA base pair |
| kb | 1000 DNA base pairs |
| TB | Transformation buffer |
| Tc | Tetracycline |
| Tp | Trimethoprim |
| Am | Ampercillin |
| Kn | Kanamycin |
| TNB | Tris NaCl Buffer |
| PCR | Polymerase Chain Reaction |
| d | Day |
| h | Hour |
| SSA | Single Strand Annealing |

| | |
|---|---|
|  | Bacterial Culture |
|  | Yeast Culture |
|  | M$^{c}$Cartney Bottle |
|  | Eppendorf tubes |
|  | 250 mL Conical Flasks |
|  | Agar plate |
|  | Centrifuge tube |
|  | Cuvette |
|  | Centrifuge |
|  | 10 µL transfer or colony transfer |
|  | Heat |
|  | Variable amount / time |
|  | Manual manipulation |
|  | Glass rod |
|  | TNB |
|  | Replica plate block |
|  | Velvet sheet |
|  | PCR Reaction |
|  | Restriction digestion |
|  | Restricted length of DNA |
|  | Plasmid |

# Abstract

Conjugation is a fundamentally important mechanism of horizontal DNA transfer between bacteria, bacteria *x* archea, and bacteria *x* eukaryotes. This work has concentrated on conjugation between bacteria *x* eukaryotes, specifically *Escherichia coli x Saccharomyces cerevisiae*. Four hypotheses were tested, investigating the barriers to this particular form of DNA transfer. The first investigated if a mutation that altered the cell-surface of the recipient *S. cerevisiae* could inhibit DNA transfer. The final three utilised a recombination-dependent-conjugation assay to investigate the barrier to DNA transmission through recombination. The hypotheses tested if the frequency of recombination, in this recombination-dependent-conjugation assay, differed when using similar or diverged DNA substrates, if a mismatch repair mutation within the recipient could affect the frequencies of recombination observed, and if the position on the plasmid of the gene of interest affected the frequency of transmission.

Transmission of the *Ura3* DNA sequence in the recipient *S. cerevisiae* was used to test all four hypotheses. The cell wall mutants *mnn9*, *knr4*, *fks1* and *kre6* were utilised to investigate if the cell-surface of the recipient could affect the frequency of transmission. The similar and diverged substrates utilised in the investigation of the affect of sequence similarity on recombination were the DNA sequences of *ura3* from *S. cerevisiae* and *Saccharomyces carlsbergensis,* respectively and the MMR mutants utilised were *msh2, pms1* and *pol30-52*. Cell wall mutants were not found to limit the frequency of transfer once donor-recipient contact was induced through the solid surface mating procedure. Sequence similarity, MMR and the relative position of the *ura3* DNA sequence on the conjugative plasmids were shown to have little effect on the frequency of transmission in *S. cerevisiae*. This suggests that any DNA that enters the nucleus of *S. cerevisiae* (eukaryotes) can recombine with the chromosome and alter it to the same extent. However, trends within the data also suggest that DNA is transferred into the recipient and then transported to the nucleus to recombine with the chromosome as a single-stranded DNA molecule.

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# Chapter 1: Introduction



The first experimental confirmation of conjugation is credited to Lederberg and Tatum (1946) who demonstrated gene transfer between genetically distinct members of *Escherichia coli*. The first similar prokaryote to eukaryote DNA transfer was reported using *Agrobacterium tumefaciens* (Chilton et al. 1977), which causes crown gall tumours in tobacco plants (Braun 1947). Today gene transfer by conjugation is known to occur between prokaryotes and representatives from all the kingdoms of life (Ferguson and Heinemann 2002). However, DNA transfer by conjugation is not without limits. Barriers to conjugation can be grouped into those that prevent DNA transfer into the recipient and those that prevent inheritance of the transferred DNA (Heinemann and Bungard 2005). This thesis reports experimental crosses between *E. coli* (prokaryote) *x Saccharomyces cerevisiae* (eukaryote) that were designed to examine both these barriers to conjugation within a eukaryotic recipient.

## 1.1 Conjugation Overview

For the purpose of this thesis, conjugation is defined as the unidirectional transfer of single-stranded DNA from a donor cell into a recipient through direct cell-cell contact (Sorensen et al. 2005). The transferred DNA in this thesis is restricted to plasmid

DNA, here defined as closed circular double-stranded DNA. These definitions are necessary because chromosomes are also capable of being transferred if mobilisable DNA is integrated into a chromosome (Cavalli-Sforza 1992), and because different definitions of plasmids are currently in use (e.g. Hinnebusch and Barbour 1991).

Conjugative plasmids require a *trans*-acting set of genes to prepare and mobilise the plasmid, and a cis-acting origin of transfer (*oriT*) (Heinemann and Bungard 2005). Different sets of *trans* acting genes have their own specific corresponding *oriT* (Ferguson and Heinemann 2002). The mechanisms by which the functions are performed by the *trans* acting gene sets differ, and are referred to as different conjugation systems, but the function of the *oriT* for each conjugation system is always, a strand-specific *cis*-acting sequence (Heineman 1991). The *oriT* is where the strand backbone is cleaved by a system-specific nickase, through hydrolysis of a single phosphodiester bond between adjacent nucleotides (Bates et al. 1998). Cell-cell contact followed by the action of the *trans* gene products on the *cis* acting *oriT* allow conjugation to occur (Heinemann 1991; Heinemann and Bungard 2005). The "nick" at the *oriT* becomes the start point for DNA transfer from the donor to the recipient (Heinemann and Bungard 2005).

The precise biochemical mechanisms that regulate conjugative transfer, beginning with the preparation of plasmid mobilisation in the donor and concluding with the conversion of the transferred DNA into an inheritable form in the recipient, has yet to be elucidated (Sorensen et al. 2005). The *trans*-acting genes of the P incompatibility group (IncP group) of plasmids are probably among the most extensively studied at the biochemical level (Bates et al. 1998). The IncP plasmids have a high frequency of transmission, can replicate in a large range of cells and can transfer into an even larger range of species than in which they can self replicate (Taylor et al. 1983; Bates et al. 1998). Because of these reasons, it was considered desirable to use the IncPα conjugation system in the majority of experiments described herein.

The *trans*-acting genes of IncP plasmids can be further sub-divided into the *tra* and *mob* gene sets (Bates et al. 1998). Conjugation begins with cell-cell contact achieved through the action of the *tra* gene-encoded pili (Bates et al. 1998; Bradley 1980; Sorensen et al. 2005). The *mob* genes encode a multi-enzyme complex denominated

the relaxosome that recognises and processes the *oriT* (Bates et al. 1998; Waters 2001). The relaxosome is then thought to facilitate 5´ directed DNA transfer into the recipient cell (Bates et al. 1998; Lanka and Wilkins 1995; Waters 2001). The DNA is currently believed to be transferred through both the donor and recipient cell surfaces by the formation and action of a pore (or 'bridge') encoded by the *tra* genes (Bates et al. 1998; Waters 2001). For a review of IncP conjugation see Zechner et al. 2000.

The IncP conjugation system is highly similar in function to Type IV secretion systems from *A. tumefaciens, Helicobacter pylori* and *Campylobacter jejuni* which mediate protein secretion and uptake (Baron et al. 2002). The genes that are currently thought to encode for the pore in both the IncP (Bates et al. 1998) and the Type IV systems (Zupan, et al. 2000) are required for conjugation. However, results of crystallographic examinations are only able to show that the Type IV pores are large enough to facilitate export of single-stranded DNA molecule out of the donor cell (Baron et al. 2002). There is, therefore, no physical evidence of DNA passing through any pore into the recipient cell (Vedantam and Hecht 2002). But transfer does occur using these systems at a very high frequency, and transfer of plasmids from prokaryotes to eukaryotes has been reported previously for both of these systems (Heinemann and Sprague Jr 1989; Chilton et al. 1977). For a review of interkingdom conjugation see (Ferguson and Heinemann 2002).

## 1.2 Recombination overview

Upon entry into the recipient, transferred plasmids must either convert back into a replicating unit, independent of the chromosome, or recombine with the chromosome by homologous recombination or by site-specific integration (Paques and Haber 1999). Plasmids are capable of being transferred into a larger range of species than they are able to replicate within (Bates et al. 1998; Mazodier and Davies 1991). Therefore, it is possible, through recombination or integration into the chromosome, for a gene to be inherited even if the plasmid will not to be able to replicate in the recipient cell (Heinemann 1991).

Homologous recombination machinery can alter an endogenous DNA sequence when two similar (homologous) but not identical substrates are combined (Harfe and Jinks-Robertson 2000; Paques and Harber 1999). Homologous regions of DNA sequence

can exist between the sequence on the transferred plasmid and another sequence within the same plasmid, within the recipient chromosome, or within any plasmids already present (Bensasson et al. 2004). The amount of sequence dissimilarity between homologous substrates is the major factor preventing homologous recombination in prokaryotes (Bensasson et al. 2004). The frequency of recombination was found to increase exponentially withincreasing sequence similarity in Bacillus, *E. coli* and in *Streptococcus pneumoniae* (Majewski and Cohan 1999; Rayssiguier et al. 1989; Majewski et al. 2000, respectively). In wild type *E. coli* cells a log-linear correlation was recently show between SOS induction and DNA divergence (Delmas and Matic 2004). Mismatch repair (MMR) enforces the sequence requirements, with deficient individuals increasing the level of dissimilarity between sequences tolerated by up to 20% (Bensasson et al. 2004). Thus there are three different types of DNA sequences: homologous, homeologous and dissimilar.

- 'Homologous', is herein defined as DNA that is similar enough to pass the constraints of both homologous recombination and MMR.
- 'Homeologous', is herein defined as DNA sequences that are similar enough to be paired by the homologous recombination apparatus, but will recombine effectively only if MMR is deactivated or temporally compromised.
- 'Dissimilar', is herein defined as DNA sequences that are too different to be paired by homologous recombination apparatus even in $MMR^-$ individuals.

Preliminary work on recombination in *S. cerevisiae* has shown the same relationships between sequence similarity and mismatch repair on intrachromosomal recombination as has been shown to be the case in prokaryotic studies (Datta et al. 1997; Surtees et al. 2004). However, the comparatively few studies on intermolecular recombination in *S. cerevisiae* have reported a much lower effect on the frequency of recombination by sequence similarity (Mezard et al. 1992; Porter et al. 1996) and little if any effect by MMR-deficient individuals (Buchanan 2002; Nicholson et al. 2006; Porter et al. 1996).

MMR is a major DNA repair system in prokaryotes and eukaryotes. Prokaryotic MMR-deficient cells have a higher mutation rate due to an inability to repair DNA

polymerase errors and an increased recombination rate (up to 1,000 fold) between homeologous sequences compared to the wild type (Funchain et al. 2001; Heinemann and Billington 2004; Zahrt and Maloy 1997). MMR deficiencies, therefore, increase the range of DNA substrates that can successfully recombine but not the amount of recombination that is initiated. Sequencing of prokaryotic MMR genes has illustrated that they have been extensively horizontally transferred over the course of evolution (Denamur et al. 2000). On the basis of these findings recombination is believed to be the driving force behind prokaryotic diversification, because any mutation within the *E. coli* genome is fifty times more likely to have occurred from recombination with DNA from another cell than by any other means (Denamur et al. 2000; Guttman and Dukuizen 1994).

## 1.3 Barriers to conjugation

For transmission of a plasmid encoded gene in a recipient exconjugant, at least two events must occur: (1) the DNA enters the recipient cell and (2) the plasmid re-forms into a double-stranded molecule (and its genes are expressed) or (3) the plasmid, or sections of the plasmid, will recombine with the chromosome by homologous recombination or by site-specific integration (Heinemann and Bungard 2005). The barriers to DNA transfer can be tested because any alteration to (1) will change the transmission frequency (observed through measuring 2 and 3). The barrier to recombination can be tested because conjugation of a plasmid into a recipient affects (3) independent of (2). Hence, the transfer barrier and recombination barrier can be investigated through different experimental designs and measuring the transmission frequencies.

### 1.3.1 Transfer Barrier

The initial barrier encountered by DNA being transferred is the surface of the recipient cell (Bates et al. 1998). Conjugation systems must facilitate the transfer of DNA through both the donor and recipient's cell wall and membrane. Theoretically, a mutation that altered the recipient's cell surface could inhibit the passage of DNA after cell-cell contact had been achieved between the donor and recipient (Skurray et al. 1974). As discussed above, the model for IncP conjugation involved the creation of a hypothetical pore in the membrane of the donor cell to facilitate transfer of the plasmid through the donor and recipient cell walls and membranes (Bates et al. 1998;

Vedamtam and Hecht 2001; Waters 2001). A mutation that resulted in a change in composition of the cell wall and/or membrane of the recipient could affect the initiation of conjugation in the donor, or ability of the pore to facilitate transfer of the plasmid into the recipient (Manning and Achtman 1979).

Putative conjugation-limiting genes (con$^-$) that are absolutely required for conjugation have never been verified (Heinemann 1991). The earliest reports of a con$^-$ mutant were in the mid 1970's (Ronald et al. 1974; Skurray et al. 1974). A mutation in the *ompA* gene of the recipient *E. coli* was initially reported as a transfer barrier for IncF plasmids transfer (Manning and Achtman 1979). OmpA is a membrane bound protein that is thought to contribute to the structural integrity of the outer membrane (Ronald et al. 1974; Wang 2002). OmpA was initially found to be the receptor for bacteriophage, then was shown to be required for IncF liquid conjugation and was then shown to be a "major target for mammalian cell defence" (Ronald et al. 1974; Wang 2002). A con$^-$ phenotype was attributed to an *ompA*$^-$ genotype because that phenotype in the recipient reduced the plasmid transmission frequency and because of its receptor activity for bacteriophage, it was suggested that OmpA was also the receptor required to initiate F plasmid conjugation (Actman et al. 1978b; Manning and Achtman 1979).

However, the *con*$^-$ phenotype of *ompA* only limited transconjugant formation in liquid mating (Achtman et al. 1978a). Transconjugants of *ompA*$^-$ and *ompA*+ recipient cells formed at equal frequencies on solid media (Manning and Achtman 1979) and microscopy showed that the *ompA* mutants aggregated in liquid media (Actman et al. 1978b; Manning and Achtman 1979; Ou and Yura 1982). The lattice of agar in the solid medium was thought to prevent aggregation of the mutants enough to prevent exclusion of the donors from the recipient aggregate (Achtman et al. 1978a). Since the conjugation frequency was not reduced on solid media, OmpA must not be the receptor molecule (Heineman 1991). The con$^-$ phenotype is, therefore, probably due to donor exclusion from aggregates of recipients that form in the liquid media, essentially limiting the amount of cell-cell contact (Heinemann 1991).

F-mediated conjugation between *E. coli* and *S. cerevisiae*, which does not have an equivalent OmpA, is consistent with the hypothesis that aggregation, and not a

specific interaction between OmpA and the donor, is the cause of the con$^-$ phenotype (Heinemann and Spargue Jr 1989). Because *S. cerevisiae* does not have an equivalent OmpA, it could not be the receptor molecule for conjugation of F plasmids and suggests that OmpA is probably not required for *E. coli x E. coli* either (although it may support a secondary stabilising function that improves the frequency of transmission) (Heinemann and Sprague Jr 1991). The vast difference in the cell wall composition between *S. cerevisiae* and *E. coli* also imply that the change in the *E. coli* cell wall due to the *ompA* mutation is not likely to have been enough to limit transfer and the observed aggregation is the more likely cause. *S. cerevisiae* transconjugants were not detected when mixed in liquid broth with donor *E. coli* (Heinemann and Sprague Jr 1989). *S. cerevisiae* transconjugants were only detected when *S. cerevisiae* and *E. coli* were mixed on solid media (Heinemann and Sprague Jr 1991). Frequencies of transmission for IncP in *E. coli x S. cerevisiae* were much lower ($2.5x10^{-7}$) (Heinemann and Sprague Jr 1989) than for *E. coli x E. coli* ($1x10^{1}$) (Ferguson et al. 2002). Conjugation between bacteria and eukaryotes is therefore possible, but is not as frequent as transfer between bacteria. However, conjugation between *E. coli x Salmonella typhimurium* is reduced to approximately the same level due to an internal temperature dependent mechanism (Heinemann 1999b). A cell surface factor may influence the frequency of conjugation into *S. cerevisiae* or internal factors such as different restriction modification regimes and transportation to the nucleus.

Hypothesis 1: A mutation altering the surface on the recipient *S. cerevisiae* cells can significantly change the transmission frequency

An isogenic *S. cerevisiae* series, comprised of four different cell wall mutants and the parental wild type, have been sourced from the Institut National des Sciences Appliquées (INSA) of Toulouse. Prior work by a visiting student indicated the possibility that two of these mutants may have a con$^-$ phenotype. The above mentioned recipient *S. cerevisiae* strains are known to have an altered cell wall composition (Lagorce et al. 2003). This is of significance because, if verified, it would be the first report of a cell surface mutation that inhibited conjugation on solid media, which is unlikely to be the result of aggregation.

As discussed above, a *con*$^-$ phenotype could alter the transmission frequency through two possible mechanisms: the cell wall composition could restrict the permeability of the *S. cerevisiae* cellular surface to DNA transfer, or a receptor on the recipient cell wall or membrane that is required for conjugation may be obscured by the altered cell wall composition. These mutants could, therefore, point toward a universal receptor molecule necessary for the initiation of conjugation and/or a less permeable composition of the cell wall or cellular membrane of the recipient.

### 1.3.2 Recombination barrier

A plasmid will re-form into a double-stranded molecule post transfer and become a unit capable of replicating independently of the chromosome, if all of its replication requirements are met in the recipient cell (Sorensen et al. 2005). Recombination can occur between the transferred plasmid and the chromosome (or another plasmid already present), whether or not the plasmid is capable of becoming a self replicating unit (Buchanan 2002). As previously mentioned in section 1.2, sequence dissimilarity between two DNA molecules seems to be the primary barrier to the initiation of homologous recombination, and the activity of MMR limits the requirements on sequence similarity further.

Two DNA molecules with regions of similarity line up and a double-stranded break in one of the regions of similarity will stimulate most recombination events (Figure 1.1) (Goldfarb and Alani 2004). Each strand at the site of the double-stranded break is degraded by exonucleases 5′ to 3′ back from the break, leaving large single-stranded overhangs (Evans and Alani 2000; Harfe and Jinks-Robertson 2000). The 3′ end of the single-stranded overhangs will then invade the corresponding homologous region in another undamaged region of similarity (i.e. in this thesis the chromosome) (Paques and Haber 1999). Invasion into the homologous region of the undamaged molecule by the 3′ ends results in the formation of a recombination intermediate (de Vries and Wackernagel 2002; Paques and Haber 1999).

There are several different intermediates that can be formed by invasion of the single-stranded overhangs into the undamaged duplex (Figure 1.1) (Paques and Haber 1999). Once the recombination intermediate is formed, DNA synthesis is initiated and primed from the 3′ end of the invading strand(s) (Evans and Alani 2000). Resolution

of the recombination intermediate can result in changes ranging from a few base pairs up to several kilobases of DNA depending on the sequence similarity between the regions of homology, and the recombination pathway (Chambers et al. 1996; Datta et al. 1996; Datta et al. 1997).

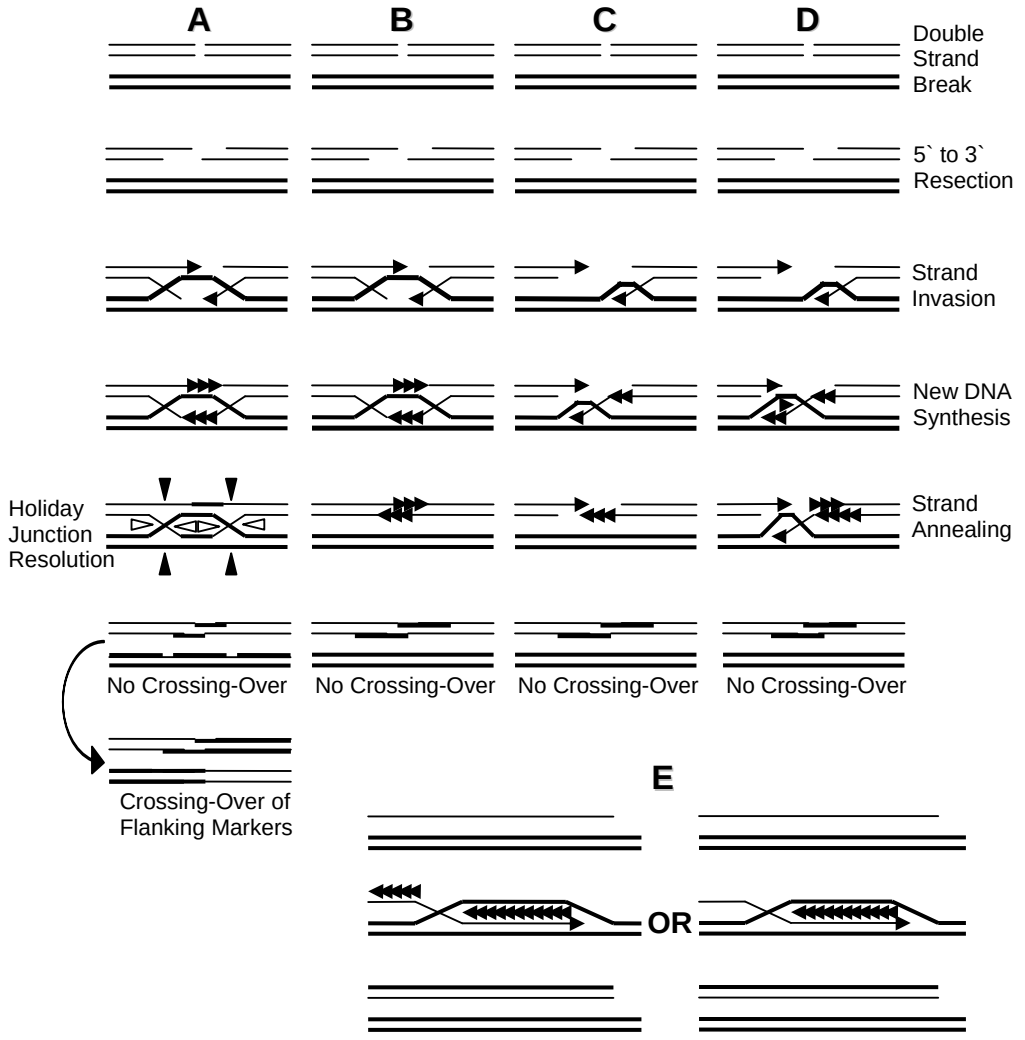



Figure 1.1: Summarised models of gene conversion from Paques and Haber (1999) (A) the DSB repair model of Szostak et al. 1983 (B) synthesis-dependent strand annealing (C) synthesis-dependent strand annealing with crossing over (D) replication fork capture (E) break induced replication. Thick lines represent the chromosome, the thin lines represent the invading strands and the arrow heads indicate DNA synthesis.

The formation and resolution of the recombination intermediate is governed by the *recABCD* genes in *E. coli* (Harfe and Jinks-Robertson 2000; Paques and Haber 1999). However, the equivalent enzymology in eukaryotes is spread over many more genes and the process of recombination is more complicated due to the increased number of

gene products (Paques and Haber 1999). The recombination enzymes are, however, not primarily responsible for enforcing the sequence similarity requirements for recombination (Surtees et al. 2004). The enzymes responsible for strictly enforcing the sequence similarity restrictions are thought to be encoded by the MMR genes (Harfe and Jinks-Roberston; Paques and Haber 1999; Surtees et al. 2004)

The particular geometry of the double helix created by complementary base pairing (i.e. AT and GC), enables MMR to proofread newly synthesised DNA by detecting warping of this conformation of the dsDNA caused by mismatches (Evans and Alani 2000; Harfe and Jinks-Robertson 2000; Hoejmakers 2001). Base pair mismatches are created by DNA polymerase errors that occur during replication or during recombination between similar but not identical substrates (Pfander et al. 2005). During recombination, MMR gene products ensure that: 1) the sequence of the 'invading' strand is similar enough to form the intermediate, 2) newly synthesised DNA primed from the invading 3´ strands is complementary to the template and 3) that newly synthesised strands of DNA are complimentary to the template after resolution of the intermediate (Chen and Jinks-Robertson 1998; Harfe and Jinks-Robertson 2000).

In order to understand how the barrier to recombination in eukaryotes will be tested in this thesis, it is necessary to give a more detailed picture of how MMR enzymes interact with DNA and other cellular processes. The way in which MMR works in prokaryotes and eukaryotes is best understood with reference to their action at the replication fork (Harfe and Jinks-Robertson 2000). A review of the replication fork will be followed by a summary of what MMR proteins are known to do during recombination. The real world examples and evolutionary implications of MMR deficiency in relation to recombination will then be discussed.

## 1.4 MMR at the Replication Fork

In *E. coli* replication, MMR becomes activated when the homodimeric MutS binds to the site of base-base mismatches or loop insertions-deletions (Figure 1.2) (Evans and Alani 2000; Harfe and Jinks-Robertson 2000). These errors result from nucleotide misincorporations or slippage errors by DNA polymerase in the newly synthesised strand (Evans and Alani 2000; Goldfarb and Alani 2004; Harris et al. 1997; Studamire

et al. 1999). MutS is able to recognise and bind to the sites of these errors by detecting conformational changes between the two strands of DNA, which are due to the incorrect binding that occurs between non-complimentary nucleotides (Harfe and Jinks-Robertson 2000).

After MutS has detected and bound to a mismatch, it will form a quaternary complex with a MutL homodimer (Evans and Alani 2000; Jiricny 2006). The binding of MutS-MutL is an ATP-dependent step which activates MutH and helicase II (UvrD). MutH is bound to the nearest upstream hemimethylated GATC site, but how activation of MutH and Helicase II actually occurs has not been experimentally demonstrated (Surtees et al. 2004). However, MutH and UvrD have both been shown to have direct interactions with the C-terminal region of MutL (Harfe and Jinks-Robertson 2000). MutH is bound to the nearest hemimethylated GATC because hemimethylation is unique to recently synthesised strands. This is because deoxyadenine methylase (Dam) lags behind the replication fork (Harfe and Jinks-Robertson 2000; Jirincy 2006). Newly synthesised strands are therefore, left transiently under-methylated because adenine at all other times of the cell cycle is methylated at these sites (Jirincy 2006; Kolodner 1995). Consequently, MutH can differentially nick the newly synthesised strand at the unmethylated GATC site (Evans and Alani 2000; Jiricny 2006; Surtees et al. 2004).

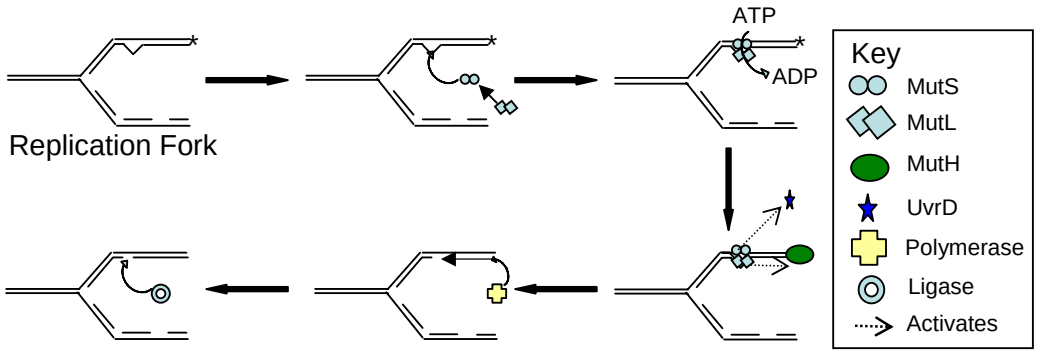



Figure 1.2: Mismatch Repair acting on a polymerase error in the leading strand of a replication fork in *E. coli.* MutS (blue circles) identifies the mismatch (drawn as a V in the newly synthesised strand) and binds to MutL (blue squares) in an ATP-dependent reaction. ATP-dependent binding of these two homodimers activates MutH and UvrD, which then cut and unwind the newly synthesised strand, respectively. Polymerase fills in and re-synthesises the strand, and then ligase seals up the remaining gaps.

Cutting only the newly synthesised strand enables helicase II (UvrD) to unwind the ends of the nicked error-containing strand from the template (Goldfarb and Alani 2004; Harfe and Jinks-Robertson 2000; Jiricny 2006). Any one of several exonucleases then digest the unwound DNA, either in the 5′ → 3′ direction (if the nearest hemi-methylated GATC site lies 5′ from the mismatch) or 3′→ 5′ (if it lies 3′ from the mismatch) (Evans and Alani 2000; Harfe and Jinks-Robertson 2000; Jiricny 2006; Kolodner 1995). Exonucleolytic degradation stops after the mismatch has been removed (Goldfarb and Alani 2004; Jiricny 2006). The resulting gap is then filled by DNA polymerase III and is completed when DNA ligase seals the remaining nick (Jiricny 2006; Surtees et al. 2004).

In the eukaryote *S. cerevisiae,* MutS and MutL have homologs, but MutH homologs are only found in gram negative bacteria such as *E. coli* (Table 1) (Harfe and Jinks-Robertson 2000). The eukaryotic MutS homolog involved in mitotic replication is a heterodimer composed of Msh2p and either Msh3p or Msh6p (Goldfarb and Alani 2004; Studamire et al. 1999; Surtees et al. 2004). The MutS homologs have been hypothesised to interact with the replication fork through associations with proliferating cell nuclear antigen (*PCNA*) (Surtees et al. 2004). The MutL homolog in mitotic replication is also a heterodimer composed of Mlh1p and Pms1p or Mlh3p (Studamire et al. 1999). Msh2p-Msh6p interacts with Mlh1p-Pms1p and initiates the repair of both single base mispairs and large regions of mispairs (Buermeyer et al. 1999). Msh2p-Msh3p interacts with both Mlh1p-Pms1p and Mlh1p-Mlh3p but can only recognise and repair regions of mispairs larger than one base pair (Chen and Jinks-Robertson 1999; Kolodner and Marsischky 1999).

Table 1.1: Homologous Mismatch repair proteins in different species

| Species | Protein | | |
|---|---|---|---|
| *E. coli* | MutS | MutL | MutH |
| *S. cerevisiae* | Msh2p-Msh3p | Mlh1p-Pms1p | n/a |
| | Msh2p-Msh6p | Mlh1p-Mlh3p | |
| *H. sapiens* | Msh2p-Msh3p | Mlh1p-Pms2p | n/a |
| | Msh2p-Msh6p | Mlh1p-Pms1p | |

*S. cerevisiae* is not methylated in the same way as *E. coli* and, therefore, no protein could replace the function of MutH in eukaryotes (Buermeyer et al. 1999; David et al. 1997), and processing of mismatches during eukaryotic replication has been

suggested to be directed by any strand break naturally present (Jiricny 2006). Suggested examples of possible strand breaks capable of differentiating the newly synthesised strand from the template strand are the 5′ or 3′ termini of Okazaki fragments or the 3` end of the leading strand (Jiricny 2006). It is currently believed that the UvrD homolog in *S. cerevisiae* is Sgs1 (Goldfarb and Alani 2004) which would be responsible for unwinding the non-complimentary strand of DNA. Differentiation between strands is thought to be possible because Sgs1p interacts with PNCA (proliferating cell nuclear antigen) to unwind the newly synthesised strand in replication (Macris and Sung 2005). Studies of *PNCA* seem to indicate that it links MMR to the replication fork and that it plays a crucial role in ensuring that the correct strand is removed (Buermeyer et al. 1999; Welz-Voegele et al. 2002).

### 1.5 MMR at the Recombination Intermediate

*S. cerevisiae* and *E. coli* MMR proteins can act as a significant barrier to the successful completion of a recombination event (Harfe and Jinks-Robertson 2000; Paques and Haber 1999). Recombination intermediates create a replication fork-like structure to synthesise the new DNA after strand invasion (Evans and Alani 2000), and as a result the replication activities of MMR also apply to the recombination intermediate (Paques and Harbour 1999). Therefore, mismatch repair will be present at sites of recombination, but the activity of the MMR gene products at the replication fork do not fully explain the activity observed during recombination in prokaryotes or eukaryotes.

In *E. coli,* MutS and MutL block homeologous strand exchange by interacting with both RecA and the DNA mismatches formed in heteroduplex DNA (Goldfarb and Alani 2004). When MMR is inactivated in prokaryotes homeologous sequences recombine at the same frequency as homologous sequences (Bensasson et al. 2004; Porter et al. 1996). In *S. cerevisiae*, the case is not clear cut. At most a 150-fold difference in homologous over homeologous recombination has been observed between intrachromosomal sequences when the MMR is active (Selva et al. 1995). This difference is reduced to 6-fold in *msh2* and 131-fold in *pms1* knockout mutants (Selva et al. 1995). So, whereas *msh2* seems to mimic the observed prokaryotic response, *pms1* seems to have little or no effect on the frequency of intra chromosomal recombination.

The role different gene products play in the formation, resolution and restrictions on the recombination intermediate are currently areas of intense research interest (Goldfarb and Alani 2004; Harfe and Jinks-Roberston 2000; Paques and Haber 1999; Sugawara et al. 2004). The formation of a recombination intermediate between two sequences that are not identical results in an invading strand that will have mismatches with the template strand. This formation is known as heteroduplex DNA (Goldfarb 2004; Alani and Studamire 1999). The formation of this heteroduplex DNA can occur through many different pathways, some of which require Msh2p-Msh3p to stabilize the intermediate and facilitate nonhomologous tail removal (Goldfarb and Alani 2004). Heteroduplex rejection involves Msh2p-Msh6p identifying the mismatches, binding ATP, but not forming a complex with the *MutL* homologs (Goldfarb and Alani 2004), instead recruiting other proteins to facilitate unwinding of the intermediate (Goldfarb and Alani 2004; Sugawara et al. 2004). Srs2p is thought to be one of these proteins, which then interacts with PNCA to unwind the heteroduplex DNA (Goldfarb and Alani 2004).

Sequence similarity is thought to play a big part in circumventing these restrictions. 20bp of continuous homology has been suggested to be needed to initiate heteroduplex formation and that 610bp of continuous homology are needed to avoid heteroduplex rejection (Datta et al. 1997). However, as previously mentioned in section 1.2 intermolecular sequences do not have the same level of sequence similarity restriction. A 6-fold difference between homologous recombination over homeologous recombination has been observed between intrachromosomal sequences when MMR is active (Porter et al. 1996). This difference is changed to 10-fold in *msh2* and 5-fold in *pms1* (Porter et al. 1996). For interchromosomal recombination Nicholson et al. (2006) found that by inactivating MMR genes they also found that recombination occurred within the same order of magnitude for wt and $MMR^-$ regardless of the DNA substrate. Subsequently, intrachromosomal recombination followed the sequence similarity and MMR behaviour observed in prokaryotes, but these same conditions have little or no effect on intermolecular recombination. So MMR gene products, and other proteins they interact with, are involved in both the formation of recombination intermediates, and interact with other gene products that are involved in sequence restriction.

## 1.6 Real world examples

The actions of the MMR proteins at the recombination intermediate are complex in eukaryotes (Paques and Haber 1999) compared to prokaryotes, where a null mutation in MMR results in an increased mutation rate and an increased substrate range for recombination (Heinemann and Billington 2004; Funchain et al. 2001; Majewski and Cohan 1999; Zahrt and Maloy 1997). The utilisation of this selective advantage in natural populations can be seen through sequencing the MMR genes, which were shown to have been extensively horizontally transferred in prokaryotes (Denamur et al. 2000). Prokaryote mutator phenotypes (individuals with an increased mutation rate) must, therefore, be present at a small proportion within every population and have a distinct advantage under conditions that require adaptation of the genome (Denamur and Matic 2006). The human body is such an environment, due to the large range of fauna, chemical and nutrient gradients prokaryotic populations are exposed to internally (Hall and Henderson-Begg 2006).

A case where increasing the range of recombination substrates lead to adaptation to an environmental stress can be seen in *Streptococcus pneumoniae*. Penicillin (a β-lactam antibiotic) was first used by Alexander Fleming in 1933 to cure Keith Rogers (MacFarlane 1984) and it only took until 1967 for the first resistant *S. pneumoniae* to be observed that had developed resistance to penicillin through altering the sequence of the penicillin binding protein genes (Dowson et al. 1989). By the late eighties, *Neisseria gonorhoeae, Neisseria meningitides, Haemophilus influenzae* and *Staphylococcus aureus* had also become resistant to penicillin through the same mechanism (Bensasson et al. 2004). Sequencing of the penicillin-resistant strains of *S. pneumoniae* showed that particular parts of the previously susceptible penicillin binding protein genes had been changed (Dowson et al. 1989). The homeologous DNA acquired from other species differed by up to 14% compared to the wild type (Dowson et al. 1989), yet at the time of examination the penicillin resistant *S. pneumoniae* had an active MMR (Humbert et al. 1995; Majewski et al. 2000).

Homeologous recombination events in *S. pneumoniae* and many other bacteria (e.g. *N. gonorhoeae, N. meningitides, H. influenzae* and *S. aureus*) that do not result in inactivation of MMR can be explained by the model described in Heinemann and

Traavik (2004) and Heinemann and Billington (2004). This model draws data from Funchain et al. (2001), who showed that individuals with an increased mutation rate (mutators) occurred spontaneously at rates of $10^{-5}$ or less in laboratory wild type populations of *E. coli*. Funchain et al. (2001) then showed that after inducing successive recombination events there was an increased proportion of mutators in the population. After the first event the proportion of mutators in the population increased from $10^{-5}$ to $10^{-2}$, and after a second recombination event more than 95% of the recombinants were $MMR^-$. Two successive recombination events therefore increased the mutator population from an initial 1/100,000 to 95/100, an increase of almost $10^5$. Heinemann and Billington (2004) argue, therefore, that a proportion of the mutators have an increased ability to take up DNA, which will enable them to adapt to the environment much faster than non-mutators.

The 'Heinemann model' extrapolates from several other key findings:

- Mutators have an increased mutation rate (from synthesis error and recombination) (Bensasson et al. 2004; Buermeyer et al. 1999);
- Mutators exist in every populations of prokaryotic cells (Denamur and Matic 2006; Hall and Henderson-Begg 2006);
- MMR deficiency allows greater recombination in prokaryotes (Denamur and Matic 2006);
- Homeologous recombination has been shown to occur *in vivo* facilitating antibiotic resistance (Dowson et al. 1989);
- The proportion of mutators in a population increases when recombination is selected (Funchain et al. 2001);

This model argues that the small proportion of mutators in a population is maintained because mutators are able to adapt to stressful environments faster than non-mutators. However, once adapted to the environmental stress, a selection favours the non-mutator phenotype. This could explain the high level of horizontal transmission of the MMR genes themselves among prokaryotes (Denamur et al. 2000). For further reading on selection against the mutator phenotype, a recent paper by Denamur and Matic (2006) summed up why mutators are selected against after adaptation.

This model describes how an existing proportion of mutators within a population would increase under environmental stress. In the case of *S. pneumoniae*, Dowson et al. (1989) and Humbert et al. (1995) suggested that the mismatch repair of *S. pneumoniae* could have been saturated which temporarily allowed homeologous recombination to occur. Bensasson et al. (2004) suggest that recombination in mutators could account for *S. pneumoniae* becoming resistant to penicillin. Resistant strains of *S. pneumoniae* did not have mutator phenotypes and MMR deficiency was not deemed a likely mechanism to gain resistance (Dowson et al. 1989). However, a recent paper has isolated a mutator strain of *S. pneumoniae* from a human clinical isolate (Hall and Henterson-Begg 2006), which may suggest that MMR-deficient phenotypes may be selected even within the human body. The 'Heinemann model' encompasses all these possibilities. A nonmutator could have temporary disabled its MMR, a nonmutator could have permanently disabled its MMR and a mutator could have adapted and then lost its mutator phenotype.

Transformation is the uptake of naked DNA from the environment and all of the options discussed above are possible for *S. pneumoniae* because it is naturally competent and may routinely take up DNA. There are many other naturally competent species, and even *E. coli* can be made competent when grown in certain environments (Bensasson et al. 2004; Claverys and Martin 2003). However, many gram negative species have gained penicillin resistance by acquiring the gene for β-lactamase (*bla)* (Bradford 2001). Plasmids carrying this gene were even detected before penicillin entered full scale production in the 1940's (Abraham and Chain 1940; Bradford 2001). Transmission (e.g. through conjugation), of *bla*-carrying plasmids between different species of bacteria will have contributed greatly to the significant resistance we see to β-lactam antibiotics (Bensson et al. 2004; Sorensen et al. 2005).

Transformation studies have been conducted in *S. cerevisiae* since the late 1970's (Hinnen et al. 1978), interchromosomal studies since the early 1980's (Jackson and Fink 1981; Klein 1984), single stranded vectors were looked at briefly in the early 1980's (Singh et al. 1982), recombination in the late 1980's and early 1990's (Dornfeld and Livingston 1992) and conjugation in the late 1980's (Heinemann and Sprague 1989). However, the vast majority of recombination work done since, has

been conducted on interchromosomal gene conversion and double stranded plasmid vectors (Nicholson et al. 2000). Bacterial competence genes do take up single-stranded DNA but the laboratory methods of transformation discussed herein transmit the DNA as a double stranded molecule.

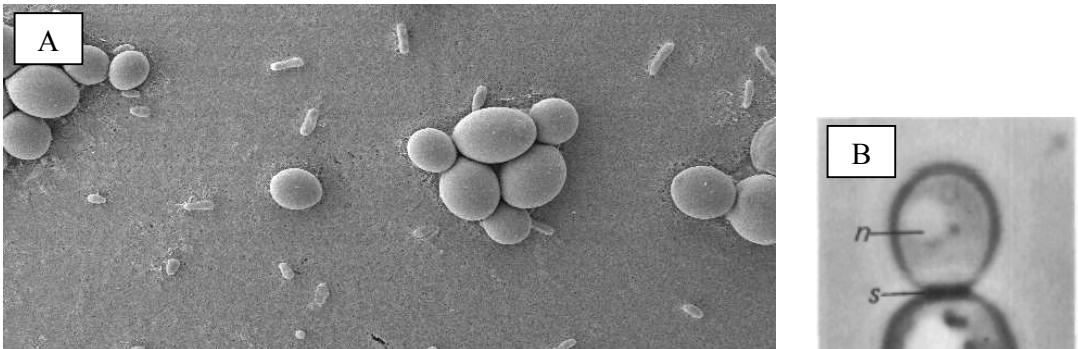
## 1.7 Evolutionary implications

The 'Heinemann model' cannot yet be applied to *S. cerevisiae* or other eukaryotes because the same key measurements of MMR on HGT have not yet been made. For this model to apply, sequence similarity would have to have an exponential effect on the frequency of recombination with horizontally transferred DNA, and MMR-deficient cells would have to significantly affect the amount of sequence dissimilarity that could recombine. This has already been shown to be true in intramolecular recombination in meiotic cells and to less of an extent in mitotic cells (Selva et al. 1995; Hoffman 1999). However, intermolecular recombination, as discussed above, has only shown an order of magnitude difference between homologous and homeologous recombination and only a very small difference in $MMR^-$ cells (Buchanan 2002; Nicholson et al. 2006; Porter et al. 1996).

Because the 'Heinemann model' explains adaptations of the chromosome through recombination or mutation, a significant effect on recombination in $MMR^-$ individuals in intermolecular recombination between horizontally transferred DNA and the chromosome needs to be seen, in order for extrapolations from prokaryotic work to apply. Intermolecular recombination with horizontally transferred DNA has so far not shown this effect, but Mezard et al. (1992) observed recombination events between plasmids, and Porter et al. (1996) observed both plasmids and chromosomal recombination events because they used plasmids that could replicate in *S. cerevisiae*. Therefore, plasmids may not be affected by MMR and sequences similarity. The Buchanan (2002) assay however, utilised a plasmid that could not replicate and would only reveal recombination events that altered the chromosome. The same results were observed for Buchanan (2002) and Porter et al. (1996) which indicates that sequence similarity has only a small effect on the intermolecular recombination frequency, and MMR has little or no effect.

The laboratory transformation differs significantly from laboratory conjugation, in that conjugation delivers plasmids in a single-stranded form into the recipient cell (Ferguson and Heinemann 2000). It is not known where or when plasmid DNA is converted back into a double-stranded molecule in *S. cerevisiae* (Figure 1.3) two possibilities exist. A transferred plasmid could be converted back into a double-stranded molecule immediately on entry into the cell (as it is in prokaryotes) or be transported as a single-stranded molecule to the nucleus where all the DNA synthesis molecules are localised in eukaryotes. This could be a significant difference because if the DNA is transported to the nucleus as a single stranded molecule, it could then recombine with the chromosome as a single-stranded molecule.

Singh et al. (1982) found that single-stranded DNA molecules partook in a recombination pathway that was not utilised by double-stranded DNA, or at least not at the same frequency. This was confirmed by Simon and Moore (1987) who concluded that "single-stranded DNA may participate directly in recombination with the chromosome". There have been no reported studies comparing homologous and homeologous recombination with MMR-deficient recipients and single-stranded DNA (Porter et al. 1996; Priebe et al. 1994). If this assay reports different results to the transformation work the DNA is different between the transformation assay and the conjugation assay, i.e. if different results to the transformation work are not found then the plasmid is a double stranded molecule when it recombines with the chromosome. Therefore, by using a plasmid that cannot replicate in the recipient, conjugation could potentially supply a new substrate (Figure 1.3), as well as restrict successful recombination to only events that result in a change to the chromosome.



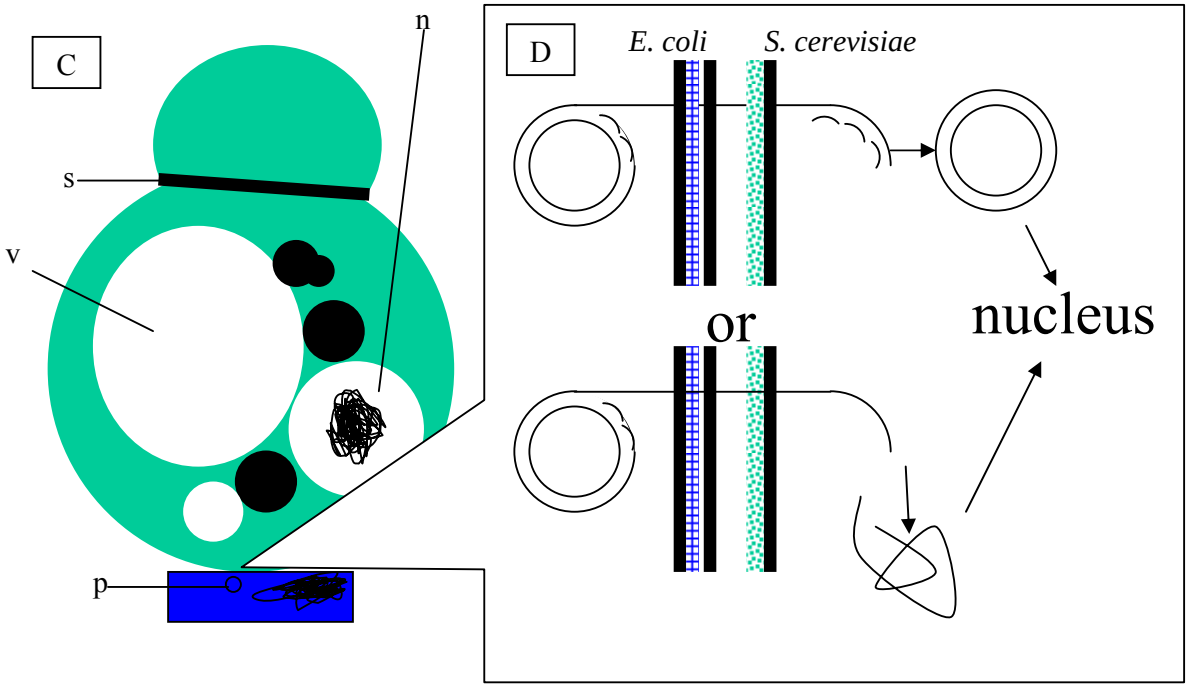



Figure 1.3: *E. coli x S. cerevisiae* conjugation: (A) Scanning electron microscope photo of *S. cerevisiae* (Large spheres) and *E. coli* (small rods), taken by the Department of Chemical Engineering at the University of Cambridge and released into the public domain. Note the size difference between *E. coli* and *S. cerevisiae*. (B) Picture from Lord and Wheals (1981), indicating various organelles in *S. cerevisiae* n – nucleus, s – septum, v – vacuole and scale line is 5µm. (C) Diagramatic representation of *S. cerevisiae x E. coli*, n – nucleus, s – septum, v – vacuole and p – plasmid. (D) Plasmid transfer through the membrane and wall of *E. coli* and then through the wall and membrane of *S. cerevisiae*. Once through the two cell surfaces there are two possible means of transportation to the nucleus, either the invading strand acts as a template and replication through okazaki fragment formation (invading strand enters by the 5´ end) synthesize a double-stranded plasmid that will be transported to the nucleus, or the single-stranded plasmid molecule is transported to the nucleus where all of the DNA synthesis machinery resides and is replicated into a double-stranded molecule.

Hypothesis 2: The frequency of recombination will be higher when homologous DNA is delivered to *S. cerevisiae* by conjugation, as compared to delivering homeologous DNA.

Hypothesis 3: The frequencies of recombination between a chromosome and either a homologous or homeologous DNA sequence on a plasmid will differ between wild type and MMR- strains.

In order to test hypothesis 2 and 3, an isogenic series of MMR-deficient *S. cerevisiae* were sourced from the Eric Alani and four plasmids were sourced from the Carlsberg Foundation, Copenhagen. The genes (homologous and homeologous) carried on these plasmids have been previously used to measure intrachromosomal recombination at a specific chromosomal locus (Hoffman 1999) in *S.cerevisiae*. The plasmids, however, were not conjugative and an *oriT* was inserted into each plasmid as described in section 4.3.1. Conjugation was then performed from *E. coli x S. cerevisiae* and selection for recombinants was performed as described in sections 4.3.3, 4.3.4 and 4.3.5. The sequences on the plasmids were homologous and homeologous to one another which enabled hypothesis 2 to be tested. The isogeneic series contains a wide range of MMR mutants which enabled hypothesis 3 to be tested. Because the *oriT* was required to be inserted, the opportunity was taken to test if the proximity of the *oriT* to gene effects the frequency of recombination.

Hypothesis 4: The position of the *oriT* will have an effect on the frequency of recombination.

As previously discussed in section 1.1, the relaxosome nicks the plasmid at the *oriT* and transfers the plasmid into the recipient from the 5′ end (Bates et al. 1998). If the plasmid is converted back into a double stranded molecule but is not recircularised, then 5′ end becomes the site of the double stranded break that will initiate recombination. The closer the double stranded break is to the gene of interest the greater the frequency of recombination (Surtees et al. 2004). Whether or not the plasmid is recircularised, the *oriT* is extremely dissimilar to gene of interest (see Chapter 4: MMR) and close proximity of extremely dissimilar sequences to the gene of interest may have an inhibitory effect on the frequency of recombination. Therefore, if the proximity of the *oriT* does effect the frequency of recombination then the nature of the effect (increase or decrease) is likely to explain the mechanism

of the effect. Therefore, by creating plasmids with *oriT* inside the gene of interest, and a 2kb away from the gene of interest, will enable hypothesis 4 to be tested.

In summary, *E. coli x S. cerevisiae* enabled the transfer and recombination barrier to be tested as outlined in subsequent chapters. The surface of the recipient was tested to look for a universal receptor molecule necessary for the initiation of conjugation and/or a less permeable composition of the cell wall or cellular membrane of the recipient. Sequence similarity is tested because intrachromosomal recombination has shown a difference in recombination between homologous and homeologous sequences especially in meiotic cells, but all intermolecular recombination has not (Porter et al. 1996; Selva et al. 1995). The effect of MMR$^-$ recipients on the frequency of recombination was tested because previous publications have observed a difference in intrachromosomal recombination but not in intermolecular (Porter et al. 1996). And finally the proximity of the *oriT* to the gene of interest was investigated in order to shed light on how this mechanism of horizontal gene transfer works in eukaryotes.

# Chapter 2: Materials and Methods



The following procedures were followed unless stated otherwise in text of subsequent chapters.

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## 2.1 Microbiological Methods

### 2.1.1 Reviving cultures

Ten micro litres (10 µL) of *S. cerevisiae* (see Table 2.1) stock, stored in 30% (v/v) glycerol (see Appendix A) at -80°C (see Appendix B), were streaked out onto solid YPD medium (Appendix A) and grown at 30°C (see Appendix B) for 36 h.

Ten micro litres of *E. coli* (Table 2.2) stock, stored in 15% (v/v) glycerol at -80°C, were streaked out onto solid LB medium (Appendix A) plus antibiotics (Appendix A) then grown at 37°C (Appendix B) for 16-18 h.

### 2.1.2 Overnight cultures

Single *S. cerevisiae* (see Table 2.1) or *E. coli* (see Table 2.2) colonies were then transferred into M$^c$Cartney bottles with liquid growth media. M$^c$Cartney bottles were pre-filled to contain the appropriate broth for growth: YPD for *S. cerevisiae* and LB for *E. coli* with the appropriate antibiotics. The cultures were then incubated at 30°C and 37°C, respectively, for 16-18 h and aerated by rotation at 200-270rpm in the Gyrotory water bath shakers (Appendix B).

NB: Occassionaly overnight cultures were inoculated straight from frozen stocks. No differences were found in the growth or characteristics of cultures grown this way.

### 2.1.3 Day cultures

Aliquots from *E. coli* overnight cultures, determined via calculations from $OD_{600}$ readings with the biophotometer (Appendix B), were transferred into 10-40 mL of day culture (dependant on the experiment). *S.* cerevisiae overnight cultures were diluted 25-fold from the overnight cultures into day cultures with no $OD_{660}$ reading taken. The composition of the day cultures were always the same as the overnight and the desired concentration of cells for the day culture, unless otherwise stated, was $1x10^7$ cells/mL for both *E. coli* and *S. cerevisiae*. *E. coli* cells were incubated 2-4 h at 37°C and rotated at 200-270rpm in the Gyrotory water bath shakers (Appendix B) to reach exponential phase ($OD_{600}$ =0.6). *S. cerevisiae* cells were incubated 6-8 h at 30°C and rotated at 200-270rpm in the Gyrotory water bath shakers to reach exponential phase ($OD_{660}$ 0.6).

2.1.4 Storing cultures

One and a half mL of overnight culture was transferred into a cryo-tube and spun-down (4000rpm for *S. cerevisiae* and 8000rpm for *E.coli* for 10 m 58010R Centrifuge F 45-30-11 rotor) in a centrifuge (Appendix B). Each tube was decanted and the pellet was resuspended in overnight culture (700 µL LB for *E.coli* and 400 µL YPD for *S. cerevisiae*) then made up to 1 mL with 50% (v/v) glycerol. Each tube was snap frozen down through submersion into liquid nitrogen (Appendix B). This snap freezing and use of glycerol minimises cell death due to ice crystal formation. All cells were maintained at -80$^{o}$C post snap freezing.

2.1.5 Antibiotics

The antibiotics[2] used in this work selected for the host *E. coli* (Table 2.2) and selected for the carriage of relevant plasmids (Table 2.3) by *E. coli*. Antibiotics were also used to segregate transconjugant, recipient and donor strains, by selective plates.

2.1.6 DNA Quantification

Solutions of DNA were quantified following the specifications of the biophotometer or the nano-rop (Appendix B). Ratios of 230nm, 260nm and 280nm were used to qualitatively identify chromosomal or RNA contamination.

2.1.7 Plating and plate preparation

Approximately 20 ml of liquefied solid LB medium or 30 ml of YPD and SC medium (Appendix A) were prepared and poured into each Petrie dish when the stock solution cooled down to approximately 40$^{o}$C once autoclaved. Antibiotics were added to LB medium and amino acids (Appendix A) were added to SC medium before pouring (Heinemann and Sprague Jr 1991). Plates were dried after pouring by removing their lids in the biohazard hood (Appendix A). Plates were used immediately or stored at 4$^{o}$C. Stored plates were given a second drying cycle in the biohazard hood before use.

Between 10 µL – 500 µL of culture were transferred via pipette onto each plate. The larger the volume of liquid to be absorbed by the medium, the drier the medium needed to be before use. If the plates were not properly dried, cells would be dispersed into a lawn, or would be distorted, by the surface moisture.

Dilution series were designed to obtain 20 – 200 colonies on a single plate. Two plates at each dilution were made and the average number of colonies was reported (see Fig 2.1). Glass beads, bent glass rod and a revolving platform were used to obtain an even spread of colonies and/or an increased speed of plating (see Fig 2.2).

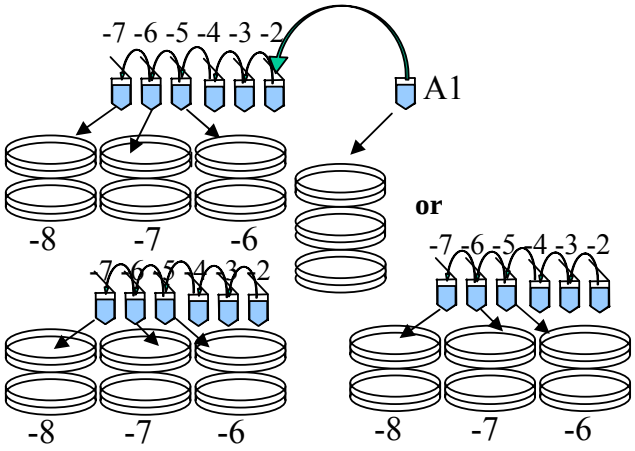


Fig 2.1. A single dilution series from the master mix goes onto three different sets of plates: Donor, Recipient and Transconjugant selective plates. Unlike in *E. coli – E. coli*, *E. coli - S. cerevisiae* crosses produce few transconjugants and must be plated directly from the master mix.

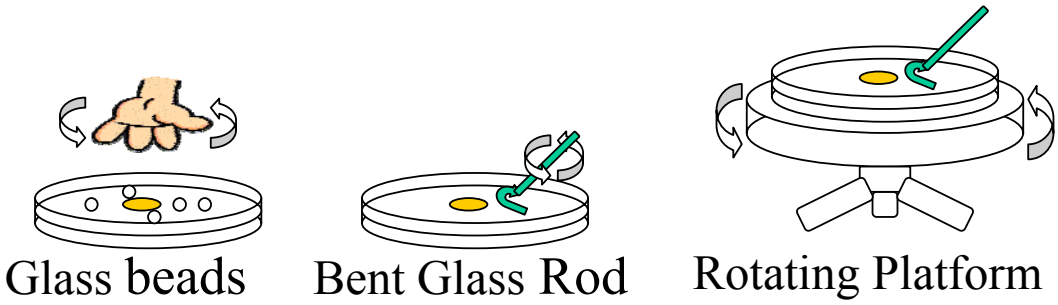


Fig 2.2. Different techniques used to spread liquid onto agar plates.

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## 2.2 Conjugation Techniques

Three different procedures were used to measure conjugation: indirect, direct and replica plating. *E. coli x E. coli* were able to be crossed using the indirect and replica plate procedures, *E.*coli x *S. cerevisiae* were able to be crossed using all three methods.

### 2.2.1 Indirect mating

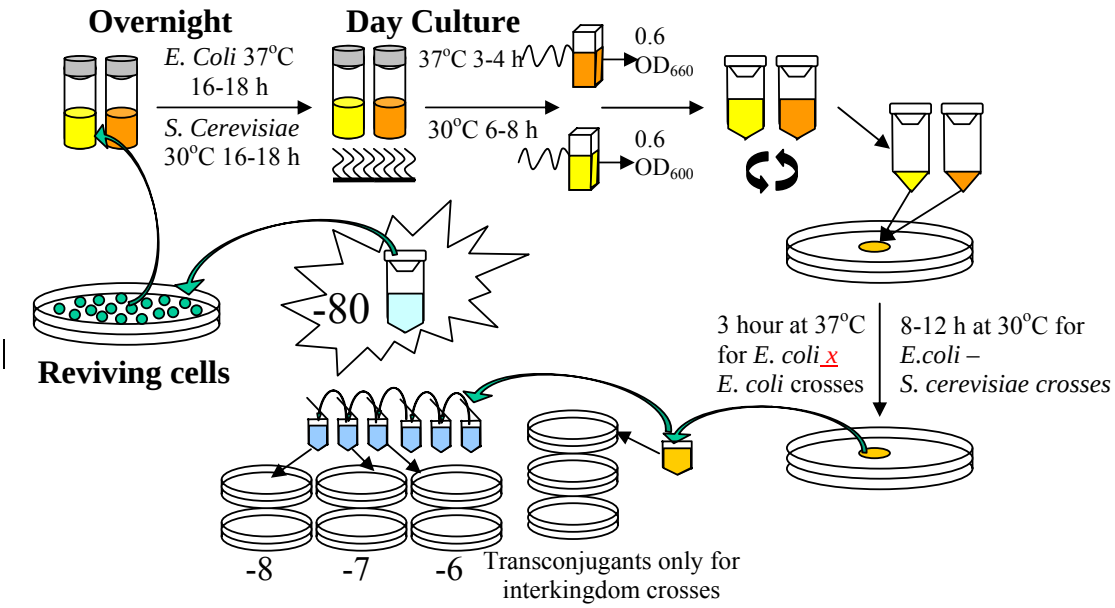


Fig 2.3. Indirect *E. coli x S. cerevisiae* mating see text below for clarification.

Indirect mating was originally described for *E.coli x S. cerevisiae* in Heinemann and Sprague Jr (1989 and 1991). The procedure used in this thesis differed slightly. Day cultures of the donors and recipients were prepared for mating. $1x10^8$ cells in the exponential phase of growth were pelleted (4000rpm for *S. cerevisiae* and 8000rpm for *E.coli* 58010R Centrifuge F 34-6-38 rotor), washed and then resuspended in 10 µL of TNB (Appendix A). *S. cerevisiae* takes longer to reach exponential phase than *E. coli,* and must be harvested at different speeds, so the first cultures to be harvested first were kept on ice until mating occurred. The resuspended 10 or 20 µL were each pipetted onto the same spot of a non-selective plate, the cells were grown for 3 h at 37°C for *E. coli x E. coli* or 8-12 h at 30°C for *E.coli x S. cerevisiae* mating. The spot was then scraped off with a wire loop and resuspended into 1 mL of TNB. Dilution

series were conducted for donor, recipient and transconjugants for *E. coli x E. coli*, but only for donor and recipients for *S. cerevisiaie* from 10 µL of the 1 mL TNB (see Figure 2.1). Plating onto *S. cerevisiae* transconjugant selective plates (SC -Ura -Thr in every experiment) differed from *E. coli x E. coli* because solution comes straight from the mating mix and not from a dilution series. 200 µL of the mating mix solution was plated onto each transconjugant selecting plate creating five plates in total. Transconjugant colonies were countered the next day from *E.coli x E. coli* crosses. Transconjugants were counted 5-15 d later for *E. coli x S. cerevisiae* crosses. The transmission frequency was calculated by dividing the number of transconjugant colonies by the number of limiting parents. The limiting parent was the donor or recipient that had the lowest concentration of cells in the mating mix (worked out through the dilution sereies).

2.2.2 Direct Mating

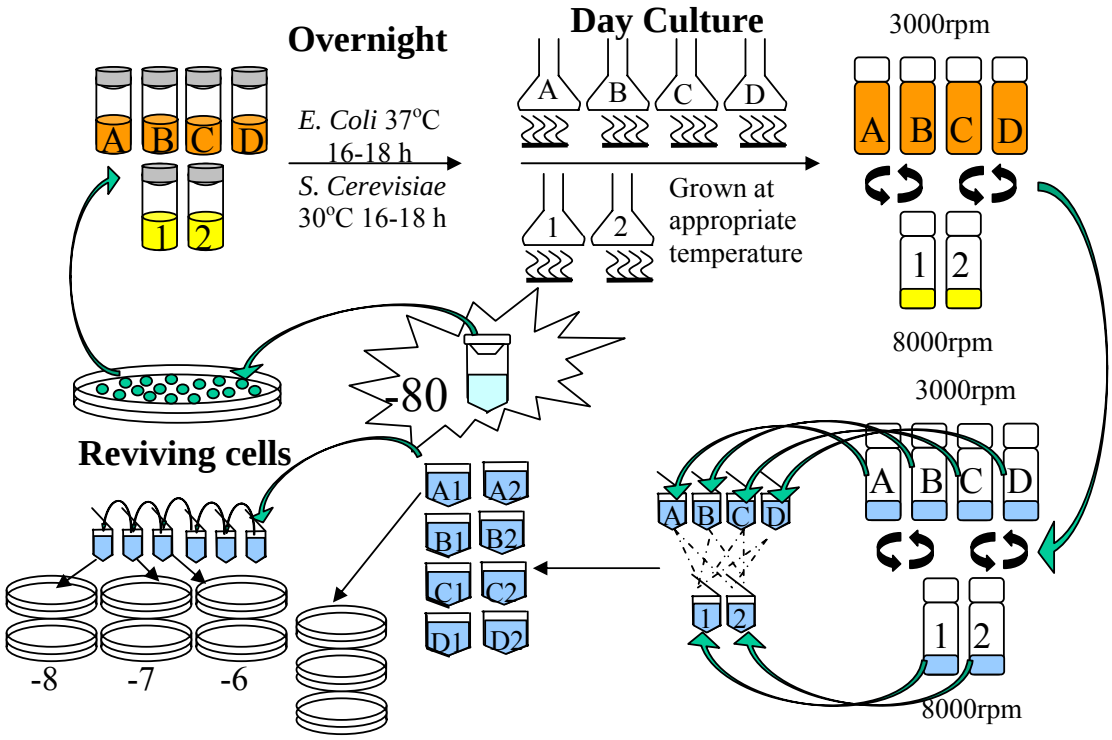


Fig 2.4. Direct *E. coli – S. cerevisiae* mating, see text below for details.

Direct mating was originally described for *E.coli x S. cerevisiae* in Heinemann and Sprague Jr (1989 and 1991). The procedure used in this thesis differed slightly. Day

cultures of the donors and recipients were prepared for mating. Once both the donor *E. coli* and recipient *S.cerevisiae* were in exponential phase cultures were placed into their respective centrifuge tubes and pelleted at 4°C for 10 m (4000rpm for *S. cerevisiae* and 8000rpm for *E.coli* 58010R Centrifuge F 34-6-38 rotor). All pellets were washed with 5 mL TNB and repelleted at the same speeds. Donor and recipients were resuspended in 0.5 mL TNB, for every 5 mL of *E. coli* culture and 25 mL of *S. cerevisiae* culture. 0.5 mL of each *E.coli* was mixed with 0.5 mL of each *S. cereviae* in separate Eppendorf tubes. Plating onto Transconjugant selective plates occurred straight from this mix. Dilution series were conducted from 10 µL of the mating mix, and then donor and recipient plates were also plated. *E. coli* plates were incubated at 37°C for 16-18 h and then counted. Recipient S. *cerevisiae* plates were incubated at 30°C for 3 d and then counted. Transconjugant plates, however, did take longer to form visible colonies and sometimes were left for up to 16 d before confirmation was obtained. The total number of cells on all five transconjugant plates was counted in order to obtain the number of transconjugants per mL of mating mix. The transmission frequency was calculated by dividing the number of transconjugant colonies by the number of limiting parents. The limiting parent was the donor or recipient that had the lowest concentration of cells/mL in the mating mix (worked out through the dilution series).

2.2.3 Replica Plating

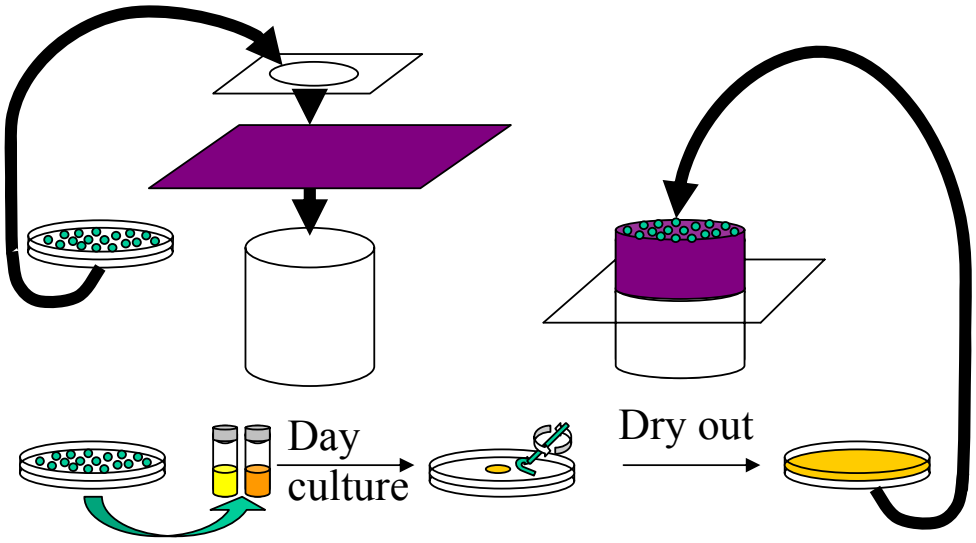


Fig 2.5. Replica plating technique (description below).

Day cultures of *E. coli* were diluted to achieve 20-200 cells per plate, and then incubated over night to allow colonies to form or plates from a transformation procedure that contain 20 – 200 colonies were used. Colonies were then transferred to velvet (Lederberg and Lederberg 1951). Day cultures of *E. coli* and/or *S. cerevisiae* were are made, transferred and spread onto transconjugant selective plates in amounts to gain a lawn. Once dry (no more than 2 m if plates were properly dried), this plate was placed onto the velvet, transferring the colonies from the velvet onto the lawn. It was found that no more than three separate day culture plates could be used before a new impression on a new sheet of velvet was required. *E. coli* plates were grown at 37$^{o}$C for 16-18 h and then counted, *S. cerevisiae* plates were grown at 30$^{o}$C for 3 days and then counted. The transmission frequency was calculated as a percentage by dividing the number of transconjugant colonies by the number of starting donor colonies.

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## 2.3 Molecular techniques

### 2.3.1 Plasmid preparation

Plasmid preparations (preps) were all conducted using the solution I, II and III basic procedure, or via the Invetrogen kit protocol[1]. When pure preparations were required then the Sambrook et al (1989) phenol/chloroform technique described in Fig 6 or the Promega kit was used. When quick preparations were required, the phenol choloroform steps were omitted and ethanol precipitation was conducted after solution III had been spun down (Birnboim and Doly. 1979).

Sambrook et al (1989) Phenol/chloroform Plasmid preparation:

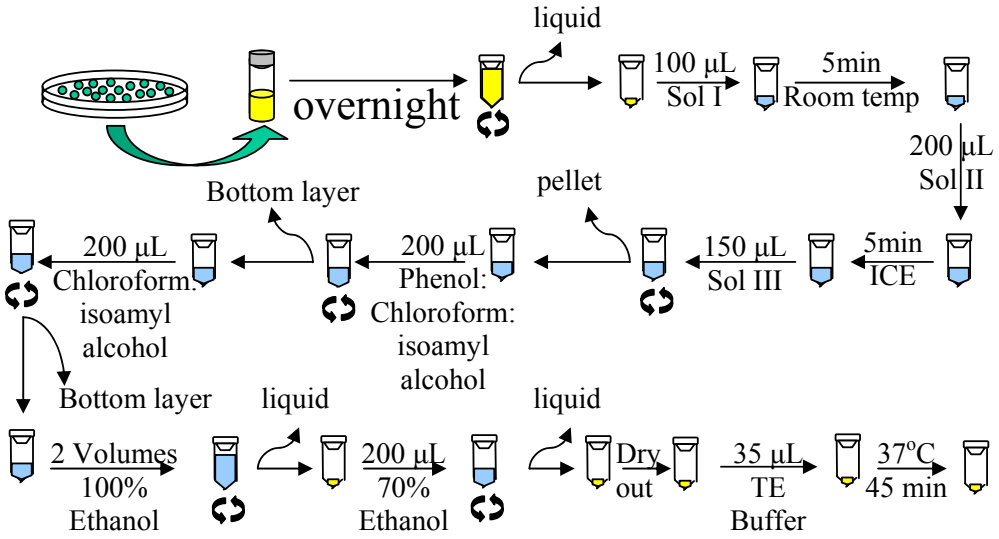


Fig 2.6. Sambrook et al (1989) phenol/chloroform plasmid preparation from *E. coli*

1.5 mL of saturated overnight cultures was pelleted at 12,000rpm 58010R Centrifuge F 45-30-11 rotor or minicentrifuge. Solutions I, II and III (Appendix A) condition the cells for lysis, differentially precipitate membranes and proteins, and introduce nicks into chromosomal DNA. The Phenol:Chloroform (Appendix A) was used to further extract residual proteins. Finally ethanol was used to precipitate the DNA. The ethanol was left to evaporate in a biosafety hood, and the DNA pellet was resuspended in TE[2]. It is important that the ethanol be removed from the final product because ethanol can inhibit subsequent reactions.

[1] http://www.invitrogen.com/content/sfs/manuals/purelink%20_quick_plasmid_qrc.pdf

2.3.2 Preparation and storage of Competent Cells

Quick prep - Sambrook et al (1989):

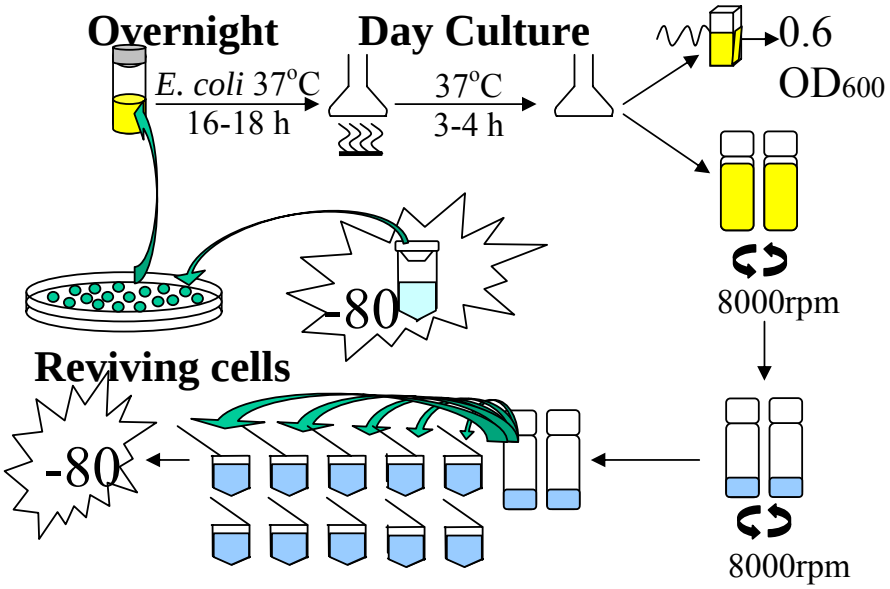


Fig 2.7. Preparing competent *E. coli* cells, see text for details.

A large volume of day culture (25-100mL) was prepared (with appropriate antibiotics). Cells in the exponential phase were pelleted by centrifugation (58010R Centrifuge F 34-6-38 rotor), resuspended in 10 mL ice cold 0.1M $CaCl_2$ (Appendix A), pelleted, and resuspended in 2ml ice cold $CaCl_2$ before being transferred into Eppendorf tubes in 200 µL aliquots. 60 µL of 50% glycerol was added to each aliquot to make the final solution up to 15% glycerol to enable freezing down of cultures for future use.

2.3.3 Highly competent cells – Inoue et al 1990:

10 – 12 large (2 – 3mm in diameter) colonies were isolated with a plastic loop, inoculated to 250 ml of SOB medium (Inoue et al 1990) in an 1800 ml Fernbach culture flask, and grown to $OD_{600}$ 0.6 at 18°C in a DAIKI incubated shaker KBLee 1001 (Appendix B). Incubation time took on average 40 h to reach $OD_{600}$ 0.6, at which time the flask was placed on ice for 10 min. The culture was then pelleted at 2500 x g (58010R Centrifuge F 34-6-38 rotor) for 10min at 4°C. The pellet was resuspended in 80ml ice cold TB (Inoue et al 1990), incubated on ice for 10min, and

repelleted. The cell pellet was gently resuspended in 20ml TB and DMSO was added up to a final concentration of 7%. After 10 m incubation on ice, the cell suspension was dispensed into 200 or 1000µL aliquots into cryotubes and then snap-frozen in liquid nitrogen. The competent cells were stored at -80$^{o}$C.

2.3.4 Transformation of *E. coli*

Competent *E. coli* were either used immediately or thawed in ice for 20 m from storage. Between 30 – 3000 µg of plasmid DNA was added to 200 µl of competent cells and incubated on ice for 20 m. Cells were then heat shocked (Appendix B) for 45-50 seconds at 42$^{o}$C and returned to ice for 2 m. Fresh LB broth was then added to a final volume of 1 mL. The culture was incubated at 37$^{o}$C in a modified (Appendix B) rotary shaker (200-270 rpm) or heatblock (300-400 rpm) for 45-90 m. Cells were then transferred onto the appropriate selective plates (Table 2.2).

2.3.5 Restriction digestion

Restriction digestions were conducted in 1.5 mL Eppendorf tubes or PCR tubes usually in 10 µL volumes. A typical restriction digestion would be as follows:

1 µL 10x buffer
1 µL Enzyme,
100 µg Plasmid DNA
4 µL $H_2O$

On occasions where a greater volume of total solution was required, $H_2O$ and 10x buffer was often all that was altered to the above formula. When more than one restriction enzyme was required, the buffer that allowed full (100%) functionality of both enzymes was chosen. Reactions were conducted at 37$^{o}$C for a minimum of 3 h. However up to 18 h was often required to ensure complete digestion of the plasmid DNA.

NB: These conditions are all well within the restrictions imposed by the Roche and Fermentas companies on their products.

2.3.6 Dephosphorylation and Ligation

To avoid plasmid recircularisation, linear plasmid DNA was dephosphorylated using Shrimp Alkaline Phosphatase (SAP). One unit hydrolyses 0.5 µmol of DNA (both

ends of a linear molecule) in 1 min at 37°C. Volumes of SAP and buffer used were dependant on the volume of solution and total amount of DNA. T4 Ligase was utilised solely at 4°C overnight for all ligation steps. One unit of enzyme and one µL of buffer were used for 10 µL of dephosphorylation and ligation reaction mix.

2.3.7 PCR

All PCR reactions were based on the program illustrated in Figure 2.8. Variables included increasing elongation time (72°C) roughly by one minute per 1000 bp of the desired fragment size and annealing temperature to increase specificity of primers. Optimization of PCR annealing temperature was conducted utilising the temperature gradient ability of the MJ mini thermal cycler (Appendix B).

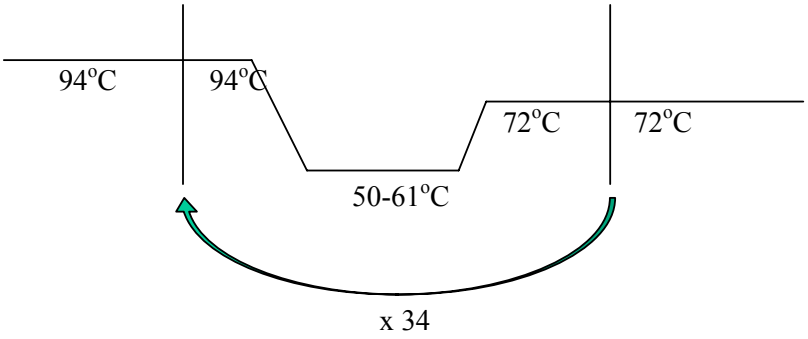


Fig 2.8. PCR program that formed the base of all PCR work done.

Reactions were normally conducted in 10 µL aliquots with the following ratios:

1 µL Buffer with out $Mg^{2+}$

0.2 µL primer L

0.2 µL primer R

0.2 µL dNTP

0.3 µL $Mg^{2+}$

1 unit Taq

$H_2O$ to 10 µL

Either DNA was added in very small amounts (0.2 µL) or an *E. coli* colony was transferred into mix.

PCR primers designed for this work through modification of pervious unpublished work:

72-inside Fwd: 5' AGTATA**AGGCCT**GCCATCCGCTTGCCCTCATC 3' (*stuI*)

72-inside Rev: 5' CCAAGC**AGGCCT**TTTCCGCTGCATAACCCTGC 3' (*stuI*)

72-outside Fwd: 5' AGTATA**TCTAGA**GCCATCCGCTTGCCCTCATC 3' (*xbaI*)

72-outside Rev: 5' CCAAGC**TCTAGA**TTTCCGCTGCATAACCCTGC 3' (*xbaI*)

78-inside Fwd: 5' CCAAGC**GTCGAC**TTTCCGCTGCATAACCCTGC 3' (*nheI*)

78-inside Rev: 5' CCAAGC**GTCGAC**TTTCCGCTGCATAACCCTGC 3' (*nheI*)

78-outside Fwd: 5' AGTATA**CATATG**GCCATCCGCTTGCCCTCATC 3' (*ndeI*)

78-outside Rev: 5' CCAAGC**CATATG**TTTCCGCTGCATAACCCTGC 3' (*ndeI*)

*ura3*-cerevisiae F 5’ CCCAACTGCACAGAACAA 3’

*ura3*-cerevisaie R 5’ TGAAGCTCTAATTTGTGAGTT 3’

*ura3*- carlsbergensis F 5’ GGTGCAAAATTCTCCAGA 3’

*ura3*- carlsbergensis R 5’ GTCTTGGTTTCGGTATACAC 3’

2.3.8 Gel Electrophoresis

Two different concentrations of agarose were used, 1% (w/v) for target bands above 1 kb and 2% (w/v) for target bands below 1 kb. TAE buffer (Appendix A) and agarose were heated in a microwave (Appendix B) until the agarose was melted. The heated solution was left to cool and then it was poured into a tray and comb mould where it was allowed to solidify. The set gel was then submerged in its tray into TAE buffer into the gel electrophoresis apparatus (Appendix B). The gels were then loaded with samples and 1 kb plus ladder was used either side of the samples as shown in fig 9. 60-90 volts were used to ensure quality of band and took 60 - 90 m to run the length at these voltages (Appendix B). Once finished the gels were stained with Ethidium bromide for 30 m and then a picture was taken using a bio-imaging gel viewer (Appendix B).

2.3.9 Gel Extractions

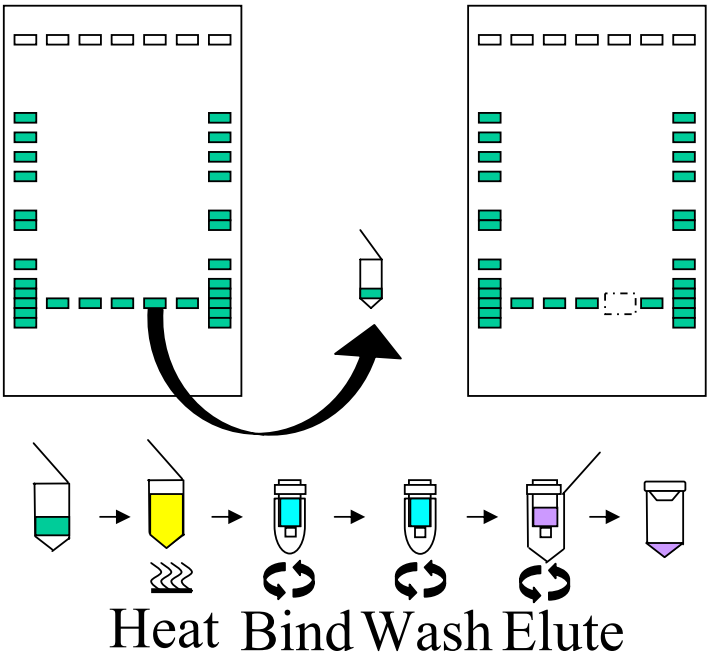


Fig 2.9. Steps required to extract a band of DNA from a gel and purify it down.

Once a gel had been developed in ethidium bromide, it was then transferred to a UV Bed (Appendix B) and the desired band was cut out using a clean sharp scalpel. This rectangle of agarose was then transferred into an Eppendorf tube where it was run through the Qiagen[2] gel extraction kit or the Promega[3] gel extraction kit (Figure 2.9). The agarose was dissolved and filtered through a column, the column bound the DNA, the DNA was washed and then eluted into a fresh sterile Eppendorf tube. This whole procedure is able to be conduced with a heating block or a water bath and a mini centrifuge.

[2] http://www1.qiagen.com/literature/handbooks/PDF/DNACleanupAndConcentration/QQ_Spin/1021422_HBQQSpin_072002WW.pdf
[8] http://www.promega.com/pnotes/82/10203_02/10203_02.pdf#search=%22promega%20gel%20extraction%20kit%22

Table 1: *S. cerevisiae*

| Strain | Genotype and/or Phenotype | Source | Reference |
|---|---|---|---|
| Cell wall mutants | | | |
| BY4741 | *MAT**a** his3Δ1 leu2Δ0 mat15Δ0 ura3Δ0*, all wall genes active | Euroscarf collection | Lagroce et al 2006 |
| Fks1 | *MAT**a** his3Δ1 leu2Δ0 mat15Δ0 ura3Δ0 Δfks1:: kan$^r$* | Euroscarf collection | Lagroce et al 2006 |
| Knr4 | *MAT**a** his3Δ1 leu2Δ0 mat15Δ0 ura3Δ0 knr4:: kan$^r$* | Euroscarf collection | Lagroce et al 2006 |
| Kre6 | *MAT**a** his3Δ1 leu2Δ0 mat15Δ0 ura3Δ0 kre6:: kan$^r$* | Euroscarf collection | Lagroce et al 2006 |
| Mnn9 | *MAT**a** his3Δ1 leu2Δ0 mat15Δ0 ura3Δ0 mnn9:: kan$^r$* | Euroscarf collection | Lagroce et al 2006 |
| SY1229 | *MAT**a** gal2Δ can1Δ his3Δ leu2Δ ura3Δ* | Heinemann collection | Heinemann and Sprague 1989 |
| MMR mutants | | | |
| EAY 235 | *ura3-52, leu2Δ1, trp1Δ63, Mat a,* | Eric Alani of Cornel University | |
| MPY 101 | *ura3-kpnI, leu2Δ1, trp1Δ63, Mat a* | Rodriguez-Beltran and Cretenet | Heinemann Collection |
| EAY 281 | *ura3-52, leu2Δ1, trp1Δ63, msh2Δ::hisG, Mat a* | Eric Alani of Cornel University | Studamire et al 1996 |
| MPY 102 | *ura3-kpnI, leu2Δ1, trp1Δ63, msh2Δ::hisG, Mat a* | Rodriguez-Beltran and Cretenet | Heinemann Collection |
| EAY 310 | *ura3-52, leu2Δ1, trp1Δ63, pms1Δ::hisG, Mat a* | Eric Alani of Cornel University | Xie et al 1998 |
| MPY 103 | *ura3-kpnI, leu2Δ1, trp1Δ63 ,pms1Δ::hisG, Mat a* | Rodriguez-Beltran and Cretenet | Heinemann Collection |
| EAY 550 | *ura3-52, leu2Δ1, trp1Δ63, pol30-52, Mat a* | Eric Alani of Cornel University | Xie et al 1998 |
| MPY 104 | *ura3-kpnI, leu2Δ1, trp1Δ63, pol30-51, Mat a* | Rodriguez-Beltran and Cretenet | Heinemann Collection |

Table 2: Bacteria

| Strain | Relevant Genotype and/or Phenotype | Source | Reference |
|---|---|---|---|
| Bacteria | | | |
| JB117 | *HsdS LleuB6, thr, r- m+, thr,* pDPT51 | Heinemann collection | Heinemann and Sprague 1989 |
| JB139 | J117 (Yep24) | Heinemann collection | |
| DE1661 | *hsdR, hsdM$^-$, met$^-$snpE$^-$, r- m+, mcrA$^-$, mcrB$^-$ gal$^-$, Δ(srlR-recA)306 ::Tn10* | Don Ennis | Heinemann 1999 |
| TB1 | *Δlac-pro rspL, ara, thi, ø80d, lacZΔM15, hsdR17 (r-, m+)* St$^r$ | Heinemann collection | |
| JB465 | TB1 (Jp116) | Heinemann collection | |
| JM109 | *recA1, endA1, gyrA96, thi, hsdR17, r$^-$, m$^+$, relA1, supE44, Δ(lac-proAB)* | Promega Cloning Kit | Yanish-Perron et al 1985 |
| XL1-blue | *recA1, endA1, gyrA96, thi, hsdR17, r$^-$, m$^+$, relA1, supE44, Δ(lac-proAB)* | Sean Devenish | Toshio et al 2000. |
| S17.1 | *thi, pro, hsdR$^-$, hsdM$^+$ ΔrecA, RP4-2-TC :Mu-Km :Tn7* | Heinemann collection | Simon, et al 1983 |
| S17.1 - 72 | S17.1 (pLH72) | | This study |
| S17.1 - 78 | S17.1 (pLH78) | | This study |
| S17.1 - 72inside | S17.1 (pLH72inside) | | This study |
| S17.1 - 72outside | S17.1 (pLH72outside) | | This study |
| S17.1 - 78outside | S17.1 (pLH78inside) | | This study |
| S17.1 - 78inside | S17.1 (pLH78outside) | | This study |

( ) = Transformed plasmid

Table 3: Plasmids

| Plasmid | Relevant Genotype and/or Phenotype | Source | Reference |
|---|---|---|---|
| pDPT51 | *tra*-R, *mob*-R, *oriT*-R, *mob*-C $Tp^r$, $Am^r$ | Heinemann collection | Youvan et al 1983 |
| YEp24 | *oriT*-C $Tc^r$, $Am^r$ *ura3* | Heinemann collection | Fukuda et al 1998 |
| F' | *tra*D36, proAB, *lac*I$^q$ZΔM15 | | Yanish-Perron et al 1985. |
| Jp116 | $Am^r$, incPα *oriT* | Heinemann collection | Simon, et al 1983 |
| pLH72 | $Am^r$, $Kn^r$, *ura3 S. cerevisiae - SalI* | Carlsberg Foundation | Hoffman thesis |
| pLH72inside | $Am^r$, $Kn^r$, *oriT,* ura3 S. cerevisiae | | This study |
| pLH72outside | $Am^r$, $Kn^r$, *oriT, ura3 S. cerevisiae* | | This study |
| pLH78 | $Am^r$, $Kn^r$, *ura3 S. carsbergensous – Δ1* | Carlsberg Foundation | Hoffman thesis |
| pLH78inside | $Am^r$, $Kn^r$, *oriT, ura3* S. *carlsbergensis* | | This study |
| pLH78outside | $Am^r$, $Kn^r$, *oriT, ura3* S. *carlsbergensis* | | This study |

# Chapter 3: Cell Surface Barrier



## 3.1 Introduction summary

DNA transmission by conjugation requires cell-cell contact between a donor bacterium containing a plasmid, along with all the necessary plasmid conjugative machinery to accomplish DNA transfer, and a recipient cell to transfer into (Heinemann 1991). The first barrier to DNA transfer is the outer physical structure of the cells themselves, particularly the membranes and walls of the donor and the recipient. If a receptor on the recipient is required to initiate conjugation, then alterations to cell-surface membrane proteins, the cell membrane, or cell wall composition could obscure that receptor and prevent DNA transfer (Skurray et al. 1974). If a receptor is not required, but the formation of the pore is dependent on the cell wall or membrane composition, then mutations that affect the cell membrane or cell wall composition will affect DNA transfer (Heinemann and Spargue 1989). However, there is currently no conclusive evidence that such mutations exist.

In the 1980's an *E. coli ompA* mutant was found to convert into a recipient exconjugant at lower frequencies than the wild type (Skurray et al. 1974). However, the observed reduced frequency was found to be a result of aggregation of recipients in liquid media (Achtman et al. 1978a), which potentially reduced access of the donors to the recipients (Heinemann 1991). When conjugation was conducted on solid media, the mutation had no effect on DNA transmission frequencies (Achtman et al. 1978b; Manning and Achtman 1979), probably because aggregates could not form as well on solid media (Heinemann 1991). This mutation therefore limited the number of

recipient cells able to contact donor cells in liquid mating mixes, not the ability of the conjugative machinery to transfer a plasmid.

Conjugation between *E. coli* and *S. cerevisiae* has only ever been observed on solid media (Heinemann 1991). The composition of the *S. cerevisiae* surface differs considerably from that of *E. coli* (Lagorce et al. 2003) and, possibly as a result, the conjugation frequencies with *S. cerevisiae* recipients are at best 10 times lower than frequencies between *E. coli x E. coli* (Heinemann and Sprague 1989). A mutation that further limits transfer by altering the composition of the cellular surface of *S. cerevisiae* is possible, and because these interkingdom crosses are only possible on solid media (Heinemann 1991), a reduced frequency would be truly relevant to the mechanism of DNA transfer from *E. coli* to *S. cerevisiae.*

Hypothesis 1: A mutation altering the surface on the recipient *S. cerevisiae* cells can significantly change the transmission frequency

## 3.2 Experimental Background

An isogenic series of four different cell wall mutants and a wild type *S. cerevisiae* (Table 2.2) were sourced from the Institut National des Sciences Appliquées (INSA) of Toulouse, to use in a hunt for cell wall mutants that affect conjugation. The wild-type *S. cerevisiae* cell wall is composed of β-1,3-glucan and β-1,6-glucan (50-60%), mannoproteins (40-50%) and chitin (2%) (Lagorce et al. 2003). These mutants have an altered cell wall composition through preventing the production of, or altering the processing of, elements in the cell wall (Lagorce et al. 2003). The resulting change in composition could possibly decrease conjugation frequency in *E. coli* to *S. cerevisiae* for reasons discussed above.

The composition of the *S. cerevisiae* cell wall is based on β-1,3-glucan and chitin forming a fibrillar network to which mannoproteins are anchored (Figure 3.1). The mannoproteins are usually anchored through β-1,6-glucan binding the proteins to β-1,3-glucan (Lipke and Ovalle 1998). However, some proteins can be directly linked to β-1,3-glucan (Lagorce et al. 2003). Mutations altering the proportion of these molecules in the cell wall disrupt the attachment of cell wall proteins that depend on the fibrillar network to anchor them into position.

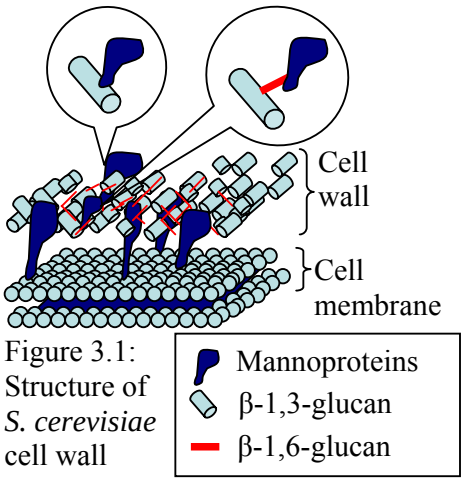


Figure 3.1: Structure of *S. cerevisiae* cell wall

The cell wall comprises 30% of the dry weight of a cell (Kapteyn et al. 1996) and all of the compositional changes induced by these mutations tend to raise the proportion of chitin (Lagroce et al. 2003). Increasing the proportion of chitin in the cell wall may also increase the rigidity of the wall and decrease its permeability to DNA or prevent formation of a pore. Alternatively, changes in the composition of the wall may obscure or remove any necessary receptor molecules or proteins required to initiate conjugation (Heinemann 1991).

Each mutation has been well characterised, but any affect on conjugation has not previously been reported. The biochemical characterisation of each mutation is described below:

- *Mnn9* encodes a subunit of the golgi membrane protein called mannosyl transferase complex. Deficiency in *Mnn9* elongates the mannan structure existent in *S. cerevisiae* extracellular proteins affecting the structure and function of the majority of extracellular proteins (Jungmann and Munro 1998).
- *Knr4* encodes a protein involved in transcriptional control of chitin synthesis, the cell wall integrity pathway and β-1,3-glucan synthesis. This mutation has been shown to increase the level of chitin in the cell wall (Fonzi 1999).
- *Fks1* encodes a subunit of the membrane bound β-1,3-glucan synthase. This mutation has been shown to increase chitin in the cell wall (probably due to the drop in β-1,3-glucan production) (Fonzi 1999).
- *Kre6* encodes a golgi membrane protein required for β-1,6-glucan synthesis (putative β-1,6-glucan synthase) (Bowen and Wheals 2004) however, a *kre6* mutation is redundant if *skn1p* is present (Roemer et al. 1994).

The cell wall mutants all have an altered cell wall composition to the wild type. The altered cell wall compositions could constitute a limitation to conjugation (Heinemann

1991). Conjugation between *E. coli* donors with recipients from this isogenic series was measured to investigate if alteration of the composition of the *S. cerevisiae* cell wall reduced the frequency of conjugation.

Three different variations of the conjugation procedure (see Section 2.2) for conducting *E. coli x S. cerevisaie* crosses have been described (Heinemann and Sprague 1991). The direct plating method was used because it gives the most accurate transmission frequencies. Because the replica plating technique uses colonies directly from a plate instead of cells and because indirect plating procedure utilises growth on non-selective medium, they can both lead to an unrepresentative number of transconjugants. In direct plating, however, each conjugation event leads to a single transconjugant colony, and therefore, does not give a misrepresentation of the transmission frequency.

All of the phenotypes of the donor, recipient and transconjugants were confirmed. YEp24 (Ura3$^{+}$) was transferred through conjugation in all of the experiments conducted in this Chapter and the transmission of the Ura3+ phenotype into the recipient was gauged through plating on selective media. *ura3* encodes a protein that is required for the production of uracil and, therefore, plating on uracil deficient media will select for transmission of the plasmid. Transmission frequencies for YEp24 have previously been reported at around 4 x $10^{-6}$ per limiting parent using the direct plating method (Heinemann and Sprague Jr 1991) and a comparable frequency was required using the same procedure before conjugation into the cell wall mutants could begin. Transmission frequencies into the INSA isogenic series using direct plating were used to test Hypothesis 1.

Particular attention was paid to the cell wall mutants *kre6* and *mnn9* because preliminary work using the indirect plating conjugation system suggested that *kre6* and *Mnn9* may reduce transmission by a factor of three compared to the wild type (Heinemann unpublished). However, as stated previously, indirect plating involves a mating step on permissive non-selective medium, and potentially allows recipient exconjugants to divide before plating. This would artificially inflate the number of transconjugants observed. Therefore, conjugation using direct plating and thoroughly

dried plates should eliminate any bias that previous work utilising indirect plating procedure may have had.

## 3.3 Results

### 3.3.1 Confirmation of the phenotypes:

The *S. cerevisiae* cell wall mutants (*wt, knr4, kre6, fks1 and mnn9*), SY1229 and the donor bacterium JB139, which is Tetracycline, Trimethoprim and Ampicillin Resistant ($Tc^r$, $Tp^r$ and $Am^r$), were plated on different selective media to confirm their reported phenotypes (Table 3.1). *S. cerevisiae* plates were incubated at 30$^o$C for 3 d and *E. coli* plates were incubated at 37$^o$C for 16-18 h. Growth of single separated colonies confirmed the phenotype. If the results were unclear fresh plates were restreaked with cells and grown again.

Table 3.1: Confirmation of phenotypes for donor and recipients through growth on selective media.

| | Media | | | |
|---|---|---|---|---|
| Strains | SC - Ura | SC - Thr | Sc - Ura - Thr | LB + Tc + Tp |
| BY4741 | - | + | - | - |
| Fks1 | - | + | - | - |
| Knr4 | - | + | - | - |
| Kre6 | - | + | - | - |
| Mnn9 | - | + | - | - |
| SY1229 | - | + | - | - |
| JB139 (YEp24) | + | - | - | + |

(- Ura) Lacing in Uracil, (- Thr) Lacking in Threonine, (+) growth and (-) no growth

### 3.3.2 *E. coli x S. cerevisiae* (JB139 *x* SY1229):

The transmission frequency of YEp24 *x* SY1229 was measured using the following media: SC - Ura - Thr (Transconjugants only), SC - Thr (Recipients + Transconjugants), and LB + Tc + Tp (Donors only). Three repetitions of the direct plating procedure were conducted following the procedure (Section 2.2.2). The negative control for this experiment involved plating both the donor and recipient onto separate transconjugant selective medium and incubation for the same length of time under the same conditions as the mating mix (12 d at 30$^o$C). The control for this experiment was the titre for donors and recipients, consistent to that of the mating

mix, plated separately on recombinant-selective medium. No colonies were observed in the negative control and transconjugant colonies were observed after 12 d on the experimental plates. The average transmission frequency of YEp24 into SY1229 was found to be $9 \times 10^{-7}$ (Table 3.2).

Table 3.2: Transmission frequencies, donor and recipient titre, and total transconjugants counted in JB139 *x* SY1229

| | Transconjugants | Donors / ml | Recipients / ml | Frequency |
|---|---|---|---|---|
| Run 1 | 51 | $5 \times 10^{8}$ | $5 \times 10^{7}$ | $1 \times 10^{-6}$ |
| Run 2 | 36 | $3 \times 10^{8}$ | $3 \times 10^{7}$ | $1 \times 10^{-6}$ |
| Run 3 | 43 | $3 \times 10^{8}$ | $7 \times 10^{7}$ | $6 \times 10^{-7}$ |

3.3.3 *E. coli x S. cerevisiae* (JB139 *x* INSA isogenic series):

The direct plating procedure was conducted as previously described above with JB139 (YEp24 ) *x* the INSA isogenic series as the recipients. No growth was observed on the negative control plates and transconjugant colonies were observed on the experimental plates after 12 d. The raw data and the plasmid transmission frequencies for each repetition of this experiment are reported in Table 3.3. The recipient was the limiting parent in every conjugation. Therefore, the transmission frequency was worked out by dividing the total number of transconjugants by the number of recipient cells per mL of mating mix. This raw data was statistically analysed and is reported in Table 3.4. The transmission frequency was found to range between 1.4 and $2.2 \times 10^{-6}$. T-tests were conducted comparing each mutant with the wild type. The T-tests conducted were two-sample equal variance with a two-tailed distribution. Values of a 95% confidence (p-value < 0.05) were considered statistically different from each other. Because there is no p-value below 0.05, not one of the mutants was statistically different from the wild type.

Table 3.3: Results table JB139 *x* INSA isogenic series containing the transmission frequencies, cell concentrations and the total transconjugants.

| Repetition 1 | Cell concentration | Transconjugants | Frequency |
|---|---|---|---|
| JB139 | $8x10^{7}$ | | |
| BY4741 | $2x10^{7}$ | 37 | $1.9x10^{-6}$ |
| *Mnn9* | $2x10^{7}$ | 36 | $1.8x10^{-6}$ |
| *Fks1* | $2x10^{7}$ | 27 | $1.4x10^{-6}$ |
| *Kre6* | 1107 | 18 | $1.8x10^{-6}$ |
| *Knr4* | $2x10^{7}$ | 32 | $1.6x10^{-6}$ |
| Repetition2 | Cell concentration | Transconjugants | Frequency |
| JB139 | $3x10^{8}$ | | |
| BY4741 | $3x10^{7}$ | 51 | $1.7x10^{-6}$ |
| *Mnn9* | $9x10^{6}$ | 27 | $3.0x10^{-6}$ |
| *Fks1* | $1x10^{7}$ | 23 | $2.3x10^{-6}$ |
| *Kre6* | $2x10^{7}$ | 34 | $1.7x10^{-6}$ |
| *Knr4* | $4x10^{7}$ | 43 | $1.1x10^{-6}$ |
| Repetition 3 | Cell concentration | Transconjugants | Frequency |
| JB139 | $1x10^{-8}$ | | |
| BY4741 | $2x10^{7}$ | 41 | $2.1x10^{-6}$ |
| *Mnn9* | $2x10^{7}$ | 33 | $1.7x10^{-6}$ |
| *Fks1* | $2x10^{7}$ | 21 | $1.0x10^{-6}$ |
| *Kre6* | $7x10^{-6}$ | 19 | $2.7x10^{-6}$ |
| *Knr4* | $2x10^{7}$ | 32 | $1.6x10^{-6}$ |

Frequency = Transconjugants divided by limiting parent, Cell concentration = per mL of mating mix.

Table 3.4: Statistical analysis of JB139 *x* INSA isogenic series Transconjugant frequencies and statistics

| Strain Name | Frequencies in each replication | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | *f* | Std Dev | p-value |
| BY4741 | $1.9x10^{-6}$ | $1.710^{-6}$ | $2.05x10^{-6}$ | $1.9x10^{-6}$ | $1.8x10^{-7}$ | |
| *Mnn9* | $2x10^{-6}$ | $3x10^{-6}$ | $1.65x10^{-6}$ | $2.2x10^{-6}$ | $7.4x10^{-7}$ | 0.4485 |
| *Fks1* | $1.4x10^{-6}$ | $2.3x10^{-6}$ | $1.05x10^{-6}$ | $1.6x10^{-6}$ | $6.5x10^{-7}$ | 0.4848 |
| *Kre6* | $1.8x10^{-6}$ | $1.7x10^{-6}$ | $2.71x10^{-6}$ | $2.1x10^{-6}$ | $5.6x10^{-7}$ | 0.5776 |
| *Knr4* | $1.6x10^{-6}$ | $1.1x10^{-6}$ | $1.6x10^{-6}$ | $1.4x10^{-6}$ | $3 x10^{-7}$ | 0.0943 |

*f* = Average Frequency, Std Dev = Standard Deviation, p = p-value assigning a value to the difference when comparing frequency means of the cell wall mutants to the wild type.

### 3.3.4 Aggregation of Cell Wall mutants:

It was observed that the two mutants *Kre6* and *Mnn9*, that in preliminary work had shown a reduced transmission frequency (Heinemann unpublished), would also aggregate in solution more than the other members of the isogenic series. In order to ascertain to what extent aggregation occured, 25 mL of day culture of each mutant was left on the bench for 30 min. A photo was taken and then the flask was agitated until all of the sediment was back in solution. After five minutes a second photo was taken. Both Kre6 and Mnn9 aggregated faster and were harder to resuspend than the other cell wall mutants (Figure 3.2).

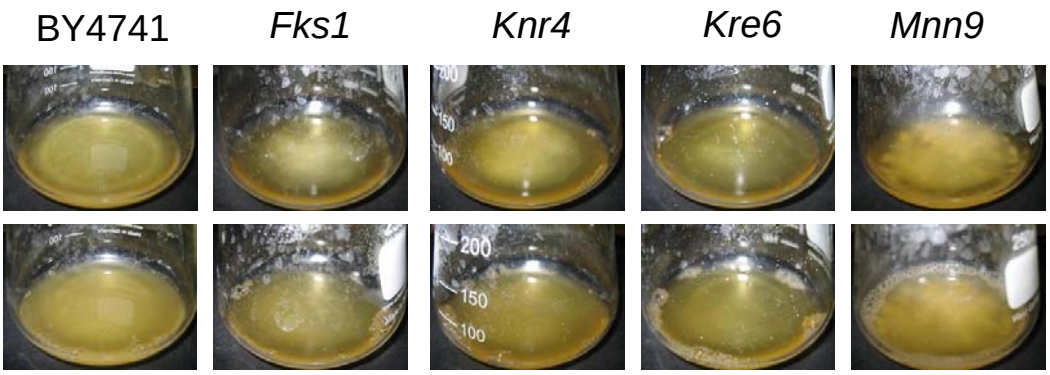


Figure 3.2: In the top row of pictures the flasks has been sitting for 30 minutes at room temperature after day culture growth. In the bottom row of pictures, the same flasks have been shaken and then a photo immediately taken. The *Kre6* and *Mnn9* required more agitation (bubbles still visible), and settled out almost immediately again. Therefore, both of these mutations seem to display an aggregation phenotype.

# Chapter 4: Mismatch Repair



## 4.1 Introduction Summary

Conjugation of *E. coli x S. cerevisiae* will be conducted to test the barriers to transmission by recombination (Outlined in section 1.7). Transmission by recombination can be tested by using plasmids that cannot replicate in *S. cerevisiae.* The DNA sequence on the plasmid and the DNA sequence in chromosome must recombine in order to create a functional gene product. This intermolecular recombination-dependent conjugation will test if alterations of the recipient MMR have an effect on the frequency of recombination and if sequence similarity between the plasmid and the chromosome has an effect on the frequency of recombination.

In *S. cerevisiae,* MMR is involved in: 1) interchromosomal recombination in the formation of the recombination intermediate, 2) ensuring faithful synthesis of the template once the intermediate has formed and 3) enforcing sequence similarity restrictions on the formation and resolution of the recombination intermediate (Goldfarb and Alani 2004; Surtees et al. 2004). Most reported work involving intrachromosomal recombination in MMR-deficient *S. cerevisiae* has revealed that the difference between homologous DNA recombination and homeologous DNA recombination decreased compared to the wild type (Porter et al. 1996; Selva et al. 1995; Surtees et al. 2004). This indicates that MMR increases the sequence similarity

requirements during homologous recombination in eukaryotes in the same way it does in prokaryotes and allows more homeologous recombination increases in the absence of fully functional MMR (Surtees et al. 2004). Porter et al. (1996) and Buchanan (2002), however, both found that the intermolecular recombination frequency of their homologous DNA molecules differed by less than an order of magnitude compared to their homeologous DNA molecules, in both the MMR mutants and the wild type. This suggests that MMR activity is influenced by whether the recombining substrates are within the same chromosome or in different molecules.

DNA transfer into eukaryotes via conjugation is complicated by the fact that the DNA probably enters the recipient cell as a single-stranded molecule and may remain in this form all the way to the nucleus where it may recombine with a homologous double-stranded DNA molecule (see Chapter 1: Introduction). Transformation with single-stranded DNA has shown that this DNA preferentially engages in a recombination pathway that is infrequently (if at all) observed with double stranded plasmids (Singh et al. 1982; Simon et al. 1983). If conjugation does result in the transfer of single-stranded DNA to the nucleus, and single-stranded plasmids do enter a different pathway to double-stranded plasmids, then sequence similarity or MMR activity may have an effect on recombination with single-stranded DNA where double stranded DNA did not (Buchanan 2002; Porter et al. 1996). Also, if recombination with single-stranded DNA does occur, then the proximity of the gene and *oriT* (which will be the ends of the DNA strand) may also have an affect on the frequency of recombination.

Hypothesis 2: The frequency of recombination will be higher when homologous DNA is delivered to *S. cerevisiae* by conjugation, as compared to delivering homeologous DNA.

Hypothesis 3: MMR-deficient *S. cerevisiae* recipients will have a different frequency of recombination with homologous and homeologous substrates acquired by conjugation compared to the wildtype.

Hypothesis 4: The position of the *oriT* will have an effect on the frequency of recombination.

## 4.2 Experimental Background

pLH71, pLH72, pLH78 plasmids were obtained from the Carlsberg Foundation, Copenhagen. The sequences of the pLH plasmids were provided by Lisa Hoffman (Ph.D. Thesis 1999) and are included in Appendix C. The pLH plasmids cannot replicate in *S. cerevisiae,* are non-conjugative and carry an inactivated allele of the uracil 3 gene from one either *S. cerevisiae* or *Saccharomyces carlsbergensis* (Chapter 2: Table 2.3). pLH71 and pLH72 both contain a *ura3* allele (homologous) originating from *S. cerevisiae,* and pLH78 contains a *ura3* allele (homeologous) originating from *S. carlsbergensis*. In order to transfer these pLH plasmids by conjugation into *S. cerevisiae,* an origin of transfer (*oriT*) must be inserted. Because the *tra* and *mob* genes act *in trans* they can be located on another plasmid or within the chromosome of the donor provided they act on a plasmid containing a compatible *oriT* (Heinemann 1991). The conjugation system IncPα was used and the corresponding *oriT* was inserted (sequence in Appendix D) into the *ura3* and outside of the *ura3* of pLH72 and pLH78 (Figure 4.1 and Figure 4.2), altering pLH72 and pLH78 into 72-in, 72-out, 78-in and 78-out respectively. 72-in, 72-out, 78-in and 78-out were created to gauge if the proximity of *oriT* to the gene of interest affects the frequency of recombination. There are two reasons why the proximity of the *oriT* may affect the frequency of recombination: 1) the ends of a molecule with a double stranded break initiate recombination and 2) no significant similarity[1] exists between the *oriT* and the sequence for *ura3* (sequences in Appendix D).

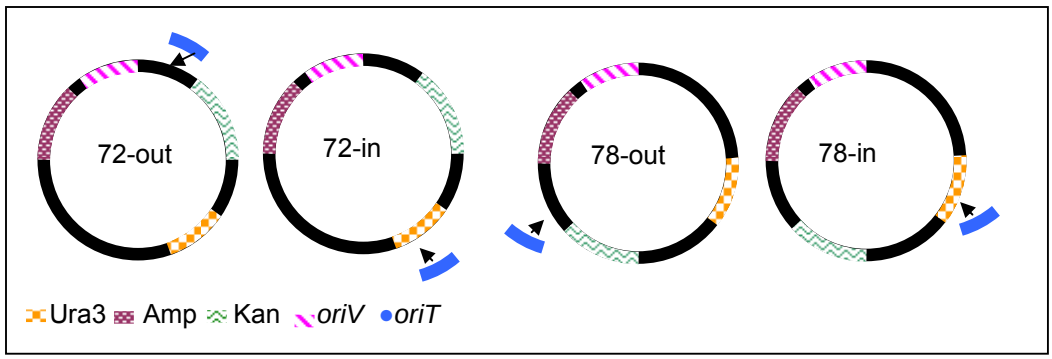


Figure 4.1: Insertion points of *oriT* into pLH72 creating 72-out (*xba*I), 72-in (*stu*I) and pLH78 creating 78-out (*nhe*I) and 78-in (*nde*I). Plasmids = 3kb, *oriT* = 0.6kb

[1] Best possible sequence alignment through blast analysis of the sequence http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi

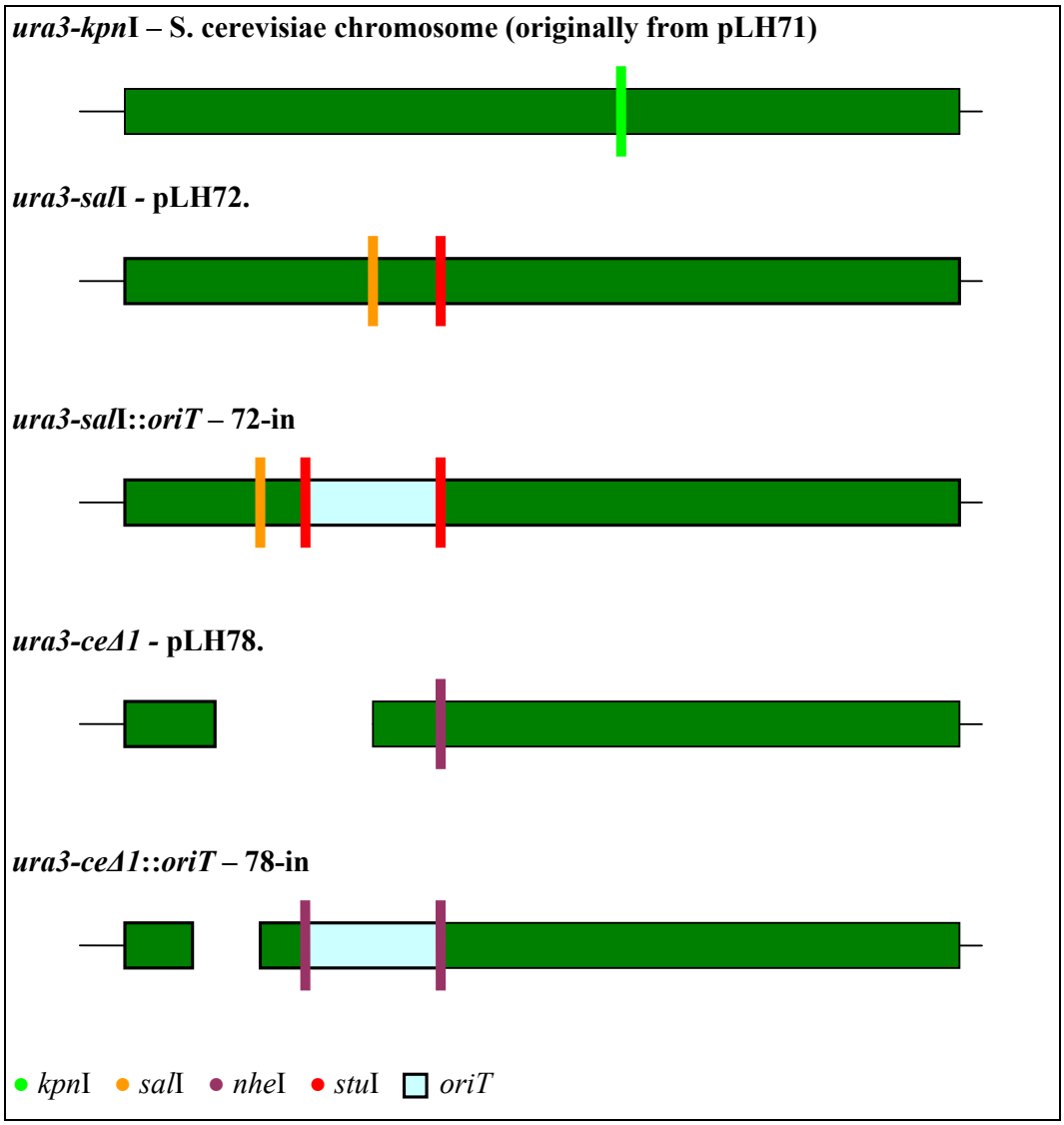


Figure 4.2: The change to the pLH72 and pLH78 *ura3* alleles through insertion of IncPα *oriT*. *oriT* = 0.6kb, *ura3-Sal*I = 0.8kb and *ura3-ceΔ1* = 0.6kb

The *ura3* DNA sequence on pLH71 and pLH72 was altered at different locations resulting in a *kpn*I and a *sal*I restriction site, respectively (Figure 4.2). The alterations to the *ura3* of both pLH71 and pLH72 created a 5bp difference (798/803 = 99.4%[1] similarity) between the pLH71 *ura3-kpn*I and pLH72 *ura3-sal*I alleles (Figure 4.3). The *ura3-Δ1* allele on pLH78 has been altered through a 125bp deletion (Figure 4.2) (Hoffman 1999), which also results in an inactive gene product. The pLH78 *ura-3Δ1* is from *S. carlsbergensis* and subsequently, has a different sequence (488/589 = 82.9%[1]) to pLH71 *ura3-kpn*I (Figure 4.4). The predicted amino acid sequences for the pLH71 *ura3-kpn*I and pLH78 *ura3Δ1* genes are 94% identical (Hoffman 1999). The

original sequences, therefore, code a similar functional gene product which is different at the DNA sequence level.

An isogenic series of Ura$^-$ *S. cerevisiae* mismatch repair (MMR) mutants were supplied by Associate Professor Eric Alani of Cornell University. The *ura3-52* allele in four of the Alani strains has been replaced with the *ura3-kpnI* sequence of pLH71. The pLH71 *ura3-kpnI* sequence was inserted by Camilo Rodriguez-Beltran and Marina Cretenet utilising the pop-in-pop-out method, described in detail in Paques and Haber (1999), (see Chapter 2: Table 2.1). The *ura3-kpnI* allele (sequence in Appendix D) can recombine with the *ura3-salI* (pLH72) and *ura3-Δ1* (pLH78) alleles to make *ura3-kpnI* functional again (Figure 4.2 and Hoffman 1999). Due to the *ura3-kpnI* insertion, recombination between incoming alleles and resident alleles can construct a *URA3* allele, enabling recombinants to be directly selected on media lacking uracil.

The threshold sequence dissimilarity considered to be homeologous in bacteria range from between <1% to ~20% (Datta et al. 1996; Dowson et al. 1989; Welz-Voegele et al. 2002). The pLH72 *ura3-salI* sequence is 0.6%[1] dissimilar to the *ura3-kpnI*, making it homologous. The pLH78 *ura3-Δ1* sequence is 17.1%[1] dissimilar to the *ura3-kpnI* making it homeologous.

[1] Best possible sequence alignment through blast analysis of the sequence
http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi
(as accessed on the 8-11-06)

```
ATGTCGAAAGCTACATATAAGGAACGTGCTGCTACTCATCCTAGTCCTGTTGCTGCCAAG  60
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ATGTCGAAAGCTACATATAAGGAACGTGCTGCTACTCATCCTAGTCCTGTTGCTGCCAAG  60

CTATTTAATATCATGCACGAAAAGCAAACAAACTTGTGTGCTTCATTGGATGTTCGTACC  120
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CTATTTAATATCATGCACGAAAAGCAAACAAACTTGTGTGCTTCATTGGATGTTCGTACC  120

ACCAAGGAATTACTGGAGTTAGTTGAAGCATTAGGTCCCAAAATTTGTTTACTAAAAACA  180
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ACCAAGGAATTACTGGAGTTAGTTGAAGCATTAGGTCCCAAAATTTGTTTACTAAAAACA  180

CATGTGGATATCTTGACTGATTTTTCCATGGAGGGCACAGTTAAGCCGCTAAAGGCATTA  240
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CATGTGGATATCTTGACTGATTTTTCCATGGAGGGCACAGTTAAGCCGCTAAAGGCATTA  240

TCCGCCAAGTACAATTTTTTACTCTTCGAAGACAGAAAATTTGCT-GACATTGGTAATAC  299
|||||||||||||||||||||||||||||||||||||||||||| | ||||||||||||||
TCCGCCAAGTACAATTTTTTACTCTTCGAAGACAGAAAATTTGGTCGACATTGGTAATAC  300

AGTCAAATTGCAGTACTCTGCGGGTGTATACAGAATAGCAGAATGGGCAGACATTACGAA  359
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AGTCAAATTGCAGTACTCTGCGGGTGTATACAGAATAGCAGAATGGGCAGACATTACGAA  360

TGCACACGGTGTGGTGGGCCCAGGTATTGTTAGCGGTTTGAAGCAGGCGGCGGAAGAAGT  419
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TGCACACGGTGTGGTGGGCCCAGGTATTGTTAGCGGTTTGAAGCAGGCGGCGGAAGAAGT  420

AACAAAGGAACCTAGAGGCCTTTTGATGTTAGCAGAATTGTCATGCAAGGGCTCCCTAGC  479
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AACAAAGGAACCTAGAGGCCTTTTGATGTTAGCAGAATTGTCATGCAAGGGCTCCCTAGC  480

TACTGGTACCATATACTAAGGGTACTGTTGACATTGCGAAGAGCGACAAAGATTTTGTTA  539
||||||  |  ||||||||||||||||||||||||||||||||||||||||||||||||||
TACTGG-AGAATATACTAAGGGTACTGTTGACATTGCGAAGAGCGACAAAGATTTTGTTA  539

TCGGCTTTATTGCTCAAAGAGACATGGGTGGAAGAGATGAAGGTTACGATTGGTTGATTA  599
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TCGGCTTTATTGCTCAAAGAGACATGGGTGGAAGAGATGAAGGTTACGATTGGTTGATTA  599

TGACACCCGGTGTGGGTTTAGATGACAAGGGAGACGCATTGGGTCAACAGTATAGAACCG  659
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TGACACCCGGTGTGGGTTTAGATGACAAGGGAGACGCATTGGGTCAACAGTATAGAACCG  659

TGGATGATGTGGTCTCTACAGGATCTGACATTATTATTGTTGGAAGAGGACTATTTGCAA  719
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TGGATGATGTGGTCTCTACAGGATCTGACATTATTATTGTTGGAAGAGGACTATTTGCAA  719

AGGGAAGGGATGCTAAGGTAGAGGGTGAACGTTACAGAAAAGCAGGCTGGGAAGCATATT  779
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AGGGAAGGGATGCTAAGGTAGAGGGTGAACGTTACAGAAAAGCAGGCTGGGAAGCATATT  779

TGAGAAGATGCGGCCAGCAAAAC  802
|||||||||||||||||||||||
TGAGAAGATGCGGCCAGCAAAAC  802
```

Figure 4.3: Sequence alignment of pLH71 *ura3-kpnI* (top) with pLH72 *ura3-salI* (bottom) using 'blast sequence alignment'
http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi
(as accessed on the 8-11-06)

```
ATGTCGAAAGCTACATATAAGGAACGTGCTGCTACTCATCCTAGTCCTGTTGCTGCCAAG  60
|||||  ||  |||||  |||||  ||||||||||||  ||||||||||  ||  ||||||||||
ATGTCTAACGCTACTTATAAAGAACGTGCTGCTGCTCATCCTAGCCCAGTTGCTGCCAAA  60

CTATTTAATATCATGCACGAAAAGCAA  87
||||  ||  ||  ||||||||||||||
TTATTAAACATTATGCACGAAAAGCAA  87


GGTAATACAGTCAAATTGCAGTACTCTGCGGGTGTATACAGAATAGCAGAATGGGCAGAC  351
|||||  |||||  ||  ||||||||||  |  ||||||||  ||  |||||  ||  |||||||||
GGTAACACAGTGAAGCTGCAGTACTCATCAGGTGTATATAGGATAGCTGAGTGGGCAGAC  153

ATTACGAATGCACACGGTGTGGTGGGCCCAGGTATTGTTAGCGGTTTGAAGCAGGCGGCG  411
||  ||  |||||  ||||||||||||||  ||  ||  |||||  |||||  |||||   |  ||  ||
ATCACCAATGCGCACGGTGTGGTGGGTCCTGGCATTGTCAGCGGGTTGAAAGAAGCCGCA  213

GAAGAAGTAACAAAGGAACCTAGAGGCCTTTTGATGTTAGCAGAATTGTCATGCAAGGGC  471
||  ||  |   ||  ||  ||  |||||||   |||  |||||  ||||||||||  ||||||||||
GAGGAGGCCACCAAAGAGCCTAGAGCTCTTCTGATGCTAGCAGAATTATCATGCAAGGGA  273

TCCCTAGCTACTGGTACCATATACTAAGGGTACTGTTGACATTGCGAAGAGCGACAAAGA  531
||  ||||||||||      ||  ||  |||||  |||||   |||||||  |||||  ||||||||
TCTCTAGCTACTGGGGAG-TACACCAAGGGCACTGTGAACATTGCCAAGAGTGACAAAGA  332

TTTTGTTATCGGCTTTATTGCTCAAAGAGACATGGGTGGAAGAGATGAAGGTTACGATTG  591
||||||||  ||  |||||  |||||||  |||||||||  ||  |||||  |||||  |||||  ||
CTTTGTTATTGGGTTTATCGCTCAAAAAGACATGGGCGGTAGAGACGAAGGGTACGACTG  392

GTTGATTATGACACCCGGTGTGGGTTTAGATGACAAGGGAGACGCATTGGGTCAACAGTA  651
||||||||||||  ||||||||  ||  ||||  ||||||||||||||||||||  ||||||||
GTTGATTATGACGCCCGGTGTAGGGCTAGACGACAAGGGAGACGCATTGGGCCAACAGTA  452

TAGAACCGTGGATGATGTGGTCTCTACAGGATCTGACATTATTATTGTTGGAAGAGGACT  711
||||||  ||||||||  ||  ||||||||  |||||  |||||  |||||  ||  ||||||||  ||
TAGAACTGTGGATGACGTAGTCTCTACTGGATCCGACATCATTATCGTGGGAAGAGGGCT  512

ATTTGCAAAGGGAAGGGATGCTAAGGTAGAGGGTGAACGTTACAGAAAAGCAGGCTGGGA  771
||||||  |||||  ||  ||||  ||  ||  ||  |||||  ||||||||||  ||||||||||
ATTTGCCAAGGGCAGAGATGTCAACGTGGAAGGTGAGCGTTACAGAAAGGCAGGCTGGGA  572

AGCATATTTGAGAAGATGCGGC  793
|||  ||  ||||  |||||||||
AGCTTACTTGAAGAGATGCGGC  594
```

Figure 4.4: Sequence alignment of pLH71 *ura3-kpnI* with pLH78 *ura3-ceΔ1* using 'blast sequence alignment' http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi (as accessed on the 8-11-06)

Work done on intrachrosomal recombination in *S. cerevisiae* using the same DNA as this work, showed that these homologous and homeologous substrates do exhibit different recombination frequencies when in the same replicon (Hoffman 1999). Recombination frequencies between the *ura3-kpnI* and *ura3-salI* were $1.8 \times 10^{-4}$ and $6.7 \times 10^{-7}$ for meiotic and mitotic recombination, respectively. Recombination frequencies for *ura3-kpnI* and the *ura3-Δ1* were $1 \times 10^{-7}$ and $1.2 \times 10^{-8}$ for meiotic and mitotic recombination respectively. Therefore, these genes have been previously been found to have different recombination frequencies in *S. cerevisiae* (Hoffman 1999) and by using these substrates in a conjugation-dependent-recombination assay, any homologous and homeologous recombination barriers imposed by sequence similarity will be observed. Conjugating into the different members of the modified isogenic series with these substrates should reveal any constraints imposed by different MMR genes.

The relevant effects of these mutations found in the isogenic series are as follows:

MPY102 (EAY281) has a complete deletion of the *msh2* gene (Studamire et al. 1996).

- Mutants have a high mutation rate and a reduced growth rate that is significantly slower than the wild type (Porter et al. 1996).
- *MSH2* is required for heteroduplex DNA removal in recombination (Studamire et al. 1999; Goldfarb and Alani 2004).
- *MSH2* is known to signal other cell cycle processes such as apoptosis and cell cycle arrest (Jirincy 2006).
- *MSH2* has been shown to be required non homologous tail removal for gene conversion in single-strand annealing (SSA) events (Sugawara et al. 1997; Studamire 1999; Goldfarb and Alani 2004).
- *msh2* has previously been shown to have a 17 fold increase on homeologous recombination compared to the wildtype in intrachromosomal recombination (Selva et al. 1995).

Therefore, the deletion of *msh2* will remove the DNA repair and antirecombinational activity of the protein and, prevent induction of apoptosis or cell cycle arrest (Jirncy 2006). This will result in an increased homeologous recombination frequency as long as a SSA-dependent recombination pathway is not required because *MSH2* is required for the non-homologous tail removal (Goldfarb and Alani 2004).

MPY103 (EAY310) has a complete deletion of *pms1* (Xie et al. 1998).

- MutLα binds to the MutS homolog in an ATP-dependent fashion activating the appropriate downstream processing steps in both replication and recombination (Welz-Voegele et al. 2002).

- In *pms1,* MutS homologs are still active and induction of other cellular processes that *MSH2* have been linked to, like apoptosis and cell cycle arrest can also still be activated (Jiricny 2006).

- The exact mechanism of the MutL homolog in replication and recombination are not known, but both require MutS binding (Welz-Voegele et al. 2002).

- *pms1* has previously been shown to have little or no effect on the frequency of recombination in intra and intermolecular recombination (Porter et al. 1996; Selva et al. 1995)

Recombination research has not found the specific effect on recombination (Porter et al. 1996), but *PMS1* has been shown to have a small effect in intrachromosomal recombination assays (Buermeyer et al. 1999).

MPY104 (EAY550) has the *pol30-52* allele inserted via a two step transplacement (Xie et al. 1998)

- *pol30-52* is a cold sensitive allele of PCNA which results in microsatellite instability and has been shown to interact with both *MSH2* and *PMS1* (Chen et al. 1999; Kokoska et al. 1999).

- It has been suggested that *PCNA* interacts with Msh2p and Pms1p to ensure preferential removal of the newly synthesised strand in recombination (Kokoska et al. 1998).

- PCNA interacts with Srs2 and blocks the *RAD52* (Pfander et al. 2005) recombination pathway, which is known to be involved in recombination between plasmid and chromosome (Dornfeld and Livingston 1991) .

Therefore, it is possible that *pol30-52* may affect the frequency of recombination between the conjugated plasmid and the chromosome. Sgs1p interacts with *PNCA* to unwind the newly synthesised strand in replication (Macris and Sung 2005) and null alleles of *sgs1* have been shown to increase the frequency of homeolgous recombination (Goldfarb and Alani 2004).

## 4.3 Results

### 4.3.1 Cloning:

The *oriT* from Jp116 was cloned into pLH72 and pLH78. Two different insertion sites on each plasmid were used to create four different plasmids: 72-in, 72-out, 78-in and 78-out (Figure 4.1). Jp116 is a pUC19 derivative, approximately 3.6kb, containing $Am^r$ and *oriT* from RK2. Unique restriction sites inside *ura3* and outside *ura3* in pLH72 and pLH78 were selected: *stu*I, *xba*I, *nde*I and *nhe*I respectively (Figure 4.1 and 3.2). Primers with these restriction sites were designed so that the primers would introduce the appropriate restriction site at either end of the PCR-amplified *oriT* fragment. The following can be seen in appendix D separated out into six small experiments with figures to assist.

The plasmid 78-out ($Am^r$ and $Kn^r$) was created first in order to optimise the procedure for cloning *oriT* (from Jp116) and inserting it into the pLH plasmids. A PCR using 78-out (*nhe*I) primers was performed following the procedure described in section 2.3.7. Both the PCR mix and pLH78 were digested with *nhe*I and then combined at the appropriate concentrations (as described in Sambrook et al. 1989) in a ligation reaction overnight (16-18 hours). S17.1 was then transformed and plated onto LB +

Tp + Am, LB + Tp + Kn and LB + Tp + Kn + Am plates. Transformants were seen on all plates. To test for the function of *oriT* a replicate plate conjugation was conducted using the colonies on transformant plates as the donors, and DE1661 as the recipient lawn, with LB + Tc + Am, LB + Tc + Kn and LB + Tc + Kn + Am as the transconjugant-selective plates. Transconjugants were only seen on LB + Tc + Am plates and only where the donor colonies were on transformant plates containing LB + Tp + Am and LB + Tp + Kn + Am. Colony PCR of the transconjugants using the 78-out (*nhe*I) primers followed by gel electrophoresis indicated that the Jp116 *oriT* was present in the transconjugants. The conjugative plasmid was approximately 3 kb in size as determined by restriction digestion and gel electrophoresis. Jp116 is 3.6 kb and pLH78 is 7 kb, so the plasmid isolated from bacteria growing on the LB + Am transconjugant plates was probably Jp116. Because Jp116 encodes the ampicillin resistance gene (*bla*) the LB + Tp + Kn selective transformant plates would not have provided selection for Jp116, which is why no transconjugants were obtained from those plates in the replica plate conjugation with DE1661. S17.1 was therefore, transformed with a mix of recircularised pLH78, which allowed growth on Kn, and the original template Jp116. pLH78 is not mobilised by the conjugative plasmid in the chromosome of S17.1,and therefore cannot be transferred into DE1661 and therefore, no transconjugant colonies were seen on plates containing Kn. In order to eliminate Jp116 from the PCR mix the amplified fragment was purified by excising it from an agarose gel as described in section 2.3.9. Gel electrophoresis and gel excision was performed on the PCR mix and the 300 bp fragment was excised and the same procedure as above was followed. This time, however, no transconjugants were observed from any plate, indicating that the gel electrophoresis and excision successfully remove Jp116 from the PCR mix.

The 300 bp *oriT* fragment and the pLH78 were digested under the same conditions and then put into the ligation mix together. Hence, the restriction digestion of either the *oriT* fragment or pLH78, or the ligation reaction was a fault. There were several possible reasons why either the restriction digestions or the ligation reaction could have been at fault: 1) pLH78 could have recircularised before the ligation reaction with *oriT*, 2) the restriction digestion did not linearise pLH78, 3) the T4 ligation enzyme was denatured, 4) exposure to UV in the gel extraction process destroyed the

function of *oriT,* or 5) the restriction sites at either end of the PCR fragment might possibly have been too close to the end for *nhe*I to hydrolyze them.

In order to circumvent these problems, the gel-excised PCR fragments were ligated into pGEM using the pGEM easy vector kit[3]. The protocol outline in that kit was followed, except that $CaCl_2$ competent DH5α were used instead of the recommended highly competent JM109. Colonies were observed on the LB + Am + X-gal + IPTG (blue/white screening) transformation plates for the PCR mix but not for the internal controls (supplied by Promega with the kit). Colony PCR of the white colonies (colonies containing recircularised pGEM were blue) indicated that the Jp116 *oriT* was present. The plasmid was 5 kb and pGEM is 3 kb in size as determined by agarose gel electrophoresis of restricted plasmid DNA. Isopropanol was found to be contaminated by conducting gel electrophoresis with all the components of the Qiagen Gel Excision Kit in separate wells. The above procedure was again performed with new isopropanol and this time there were no colonies on any of the transformation plates.

Because there were no colonies on the internal control plates, I hypothesised that the problem was with the transformation frequency. So the above procedure was followed again, but this time with JM109 highly competent cells. Blue and white colonies were observed on all of the transformation plates including the controls. Colony PCR indicated that an *oriT* was present. Restriction digestion of plasmid isolate with *nhe*I and pGEM-specific *sca*I was conducted to prevented recircularisation of pGEM and enabled visualisation of the digested *oriT* fragment, after separation by gel electrophoresis (Figure 4.5). After gel electrophoresis the digested *oriT* DNA fragment was then excised from the gel. pLH78 was digested with *nhe*I and dephosphorylated with SAP in the same reaction, but was not inactivated. A ligation reaction containing digested *oriT* and linearised pLH78 then combined at the appropriate concentrations (Sambrook et al. 1989) in a ligation reaction overnight. $CaCl_2$ competent S17.1 cells were then transformed with the ligation mix and plated on LB + Tp + Am + Kn, LB + Tp + Am and LB + Tp + Kn plates (to select for 78-out transformants $Am^r$ and $Kn^r$). No colonies were observed using these cells, so highly

[3] http://www.promega.com/tbs/tm042/tm042.pdf
(as accessed on the 8-11-06)

competent S17.1 cells were prepared and transformed with the ligation mix. Transformants were observed using the highly competent S17.1 cells. A replicate plate conjugation was conducted using the colonies from the transformant plates as the donors and DE1661 as the recipient lawn, with LB + Tc + Am, LB + Tc + Kn and LB + Tc + Kn + Am as the transconjugant selective plates. Transconjugants were observed on all plates. Gel electrophoresis of colony PCR and plasmid isolation confirmed the presence of *oriT* and the transconjugant plasmid as 7kb in size.

Highly competent S17.1 cells were again transformed, this time with the isolated plasmid from a transconjugant colony and labelled 78-out. The optimised procedure is therefore: PCR, gel electrophoresis, gel excision, pGEM cloning kit using highly competent JM109 (or XL1blue), plasmid isolation, restriction digestion, gel excision, ligation of digested *oriT* into a linearised and dephosphorylated pLH plasmid, transform highly competent S17.1 cells with ligation mix, replica plate conjugate the transformant colonies DE1661, isolate plasmids and transform S17.1. The other three plasmids were then constructed using the optimised procedure along with *nhe*I as a positive control for each step (Figure 4.6 and Figure 4.7).

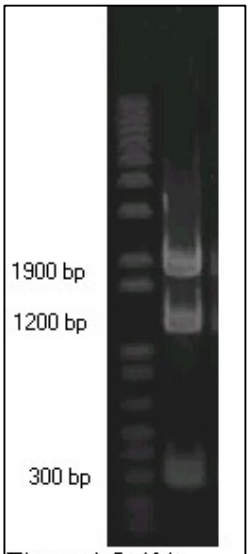


Figure 4.5: 1% agarose gel digested with nheI and scaI

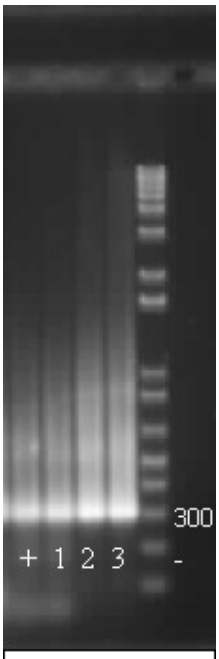


Figure 4.6: 2% gel, +n*he I*, 2 *xba I*, 3 *stu I*, 4 *nde I*, - noDNA

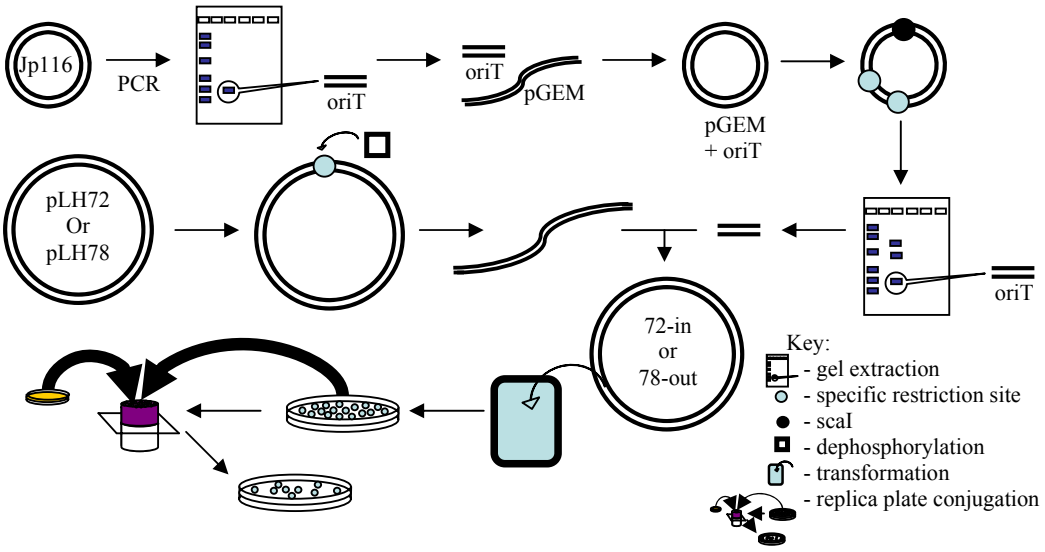


Figure 4.7: Overview of the cloning of *ori*T into two different plasmids.

### 4.3.2 *E. coli x E. coli* conjugation

Conjugation between *E. coli x E. coli* was conducted in order to make quantitative measurements of each donor's transmission frequency. The above cloned donor plasmids have had the *oriT* from an RK2 (in Jp116) inserted into them. S17.1 ($Tp^r$) contains a portion of the plasmid RK2, which contains the IncPα *tra* and *mob* gene sets, integrated into the chromosome (see Chapter 2: Table 2.2). IncPα plasmids such as RK2 and RP4 commonly have a transmission frequency just below one (Ferguson et al. 2002) and these are expected to be the same.

A indirect plate conjugation (see section 2.2.1) was conducted using 72-in, 72-out, 78-in and 78-out (all $Am^r$ and $Kn^r$) as the donor and DE1661 ($Tc^r$) as the recipient. The conjugation was conducted using the following media: LB + Tc + Am + Kn (transconjugant), LB + Tp (donor) and LB + Tc (recipient). The controls used were 1) S17.1 (pLH72) and S17.1 (pLH78) *x* DE1661 following the procedure above, 2) S17.1 *x* DE1661 onto LB + Tc + Tp (Transconjugant), LB + Tc (Recipient) and LB + Tp (Donor) plates and 3) Each donor and the recipient were plated alone on the transconjugant selection plates. No transmission of pLH72, pLH78 and integrated RK2 was observed, and no recipient or donor grew on the transconjugant selective plates. The frequencies of the transmission are shown in Table 4.1.

Table 4.1: Frequency of transmission of *E. coli x* DE1661

| Strain | Donor (D) Cells/ml | Recipient (R) Cells/ml | Transconjugants (T) Cells/ml | Frequency T/LP |
|---|---|---|---|---|
| 72-in | $2.0 \times 10^7$ | $1.0 \times 10^8$ | $1.0 \times 10^6$ (140)* | 0.05 |
| 72-out | $1.0 \times 10^7$ | $1.0 \times 10^8$ | $1.0 \times 10^6$ (106) | 0.10 |
| 78-in | $4.0 \times 10^7$ | $1.0 \times 10^8$ | $1.0 \times 10^7$ (182) | 0.25 |
| 78-out | $5.0 \times 10^7$ | $2.0 \times 10^8$ | $1.0 \times 10^7$ (229) | 0.25 |
| pLH72 | $4.0 \times 10^7$ | $1.0 \times 10^8$ | 0 | $\leq 2.5 \times 10^{-8}$ |
| pLH78 | $2.0 \times 10^7$ | $1.0 \times 10^8$ | 0 | $\leq 5.0 \times 10^{-8}$ |
| S17.1 | $1.0 \times 10^7$ | $9.0 \times 10^8$ | 0 | $\leq 1.0 \times 10^{-8}$ |

Limiting parent (LP), in this case the limiting parent was always the donor. * Numbers in brackets are the number of transconjugants counted.

4.3.3 *E. coli x S. cerevisiae* conjugation:

A direct plating conjugation between S.17.1 with 72-in, 72-out, 78-in or 78-out *x* MPY101, MPY102, MPY103 or MPY104 (*wt, msh2, pms1* or *pol30-52,* respectively) was conducted (see section 2.2.2). Because the colonies observed result from a recombination event, the colonies observed will be called recombinants. All 16 different combinations of donors *x* recipients crosses were conducted at the same time for all three repetitions of this assay. For each combination, 25 mL of *S. cerevisiae* day culture and 10 mL of *E. coli* were concentrated into a single combined mL of mating mix. Therefore, day cultures consisting of 100ml YPD for each *S. cerevisiae* and 40ml LB + Am + Kn for each *E. coli*, were grown and then partitioned off into quarters. The media used was: SC – Ura – Thr (recombinants only), LB + Am (donors only) and SC – Thr (recipient + recombinants). Controls conducted were, 1) donors and recipients titre, consistent to that of the mating mix, plated separately on recombinant-selective media and 2) S17.1 (pLH72) and S17.1 (pLH78) *x* the MMR mutants. No colonies were observed on any of the control plates. Table 4.2 reports the raw data (concentration of donors and recipients, and the number of recombinant colonies) of the three repetitions conducted. The frequency of each combination for each repetition was calculated (Table 4.3). The mean (Table 4.4) and standard deviation (Table 4.5) from the data in Table 4.3 has been calculated and tabulated for each combination. The standard error has then been calculated (Table 4.6) and then combined with the mean recombination frequency (Table 4.8). The p-values

comparing each mutant to the wildtype (Table 4.9) for each donor plasmid and each donor plasmid in each mutant (Table 4.10) were then calculated and tabulated.

Table 4.2: Titres of donors and recipients, and the recombinant colony number per repetition of S.17.1 with 72-in, 72-out, 78-in or 78-out *x wt, msh2, pms1* or *pol30-52*

| Repetition 1 | *wt* | *msh2* | *pms1* | *Pol30-52* | Donor titre |
|---|---|---|---|---|---|
| 72 - in | 48 | 1 | 15 | 34 | $1x10^8$ |
| 72 - out | 32 | 1 | 21 | 21 | $8x10^7$ |
| 78 - in | 26 | 1 | 18 | 35 | $5x10^6$ |
| 78 - out | 40 | 1 | 28 | 34 | $1x10^7$ |
| Recipients titre | $1x10^8$ | $1x10^7$ | $1x10^8$ | $5x10^7$ | |

| Repetition 2 | *wt* | *msh2* | *pms1* | *pol30-52* | Donor titre |
|---|---|---|---|---|---|
| 72 - in | 7 | 2 | 5 | 9 | $1x10^7$ |
| 72 - out | 2 | 1 | 3 | 17 | $1x10^7$ |
| 78 - in | 7 | 1 | 5 | 17 | $1x10^7$ |
| 78 - out | 4 | 1 | 4 | 10 | $1x10^7$ |
| Recipients titre | $1x10^8$ | $2x10^7$ | $8x10^7$ | $4x10^7$ | |

| Repetition 3 | *wt* | *msh2* | *pms1* | *pol30-52* | Donor titre |
|---|---|---|---|---|---|
| 72 - in | 7 | 2 | 5 | 7 | $5x10^7$ |
| 72 - out | 3 | 1 | 3 | 5 | $8x10^7$ |
| 78 - in | 7 | 1 | 8 | 6 | $7x10^7$ |
| 78 - out | 4 | 1 | 4 | 6 | $5x10^7$ |
| Recipients titre | $3x10^7$ | $1x10^7$ | $5x10^7$ | $8x10^6$ | |

Donor and recipient titres are reported in cells/mL in the mating mix (calculated from the dilution series).

Table 4.3: Frequency of transmission (recombination)

| Repetition 1 | *wt* | *msh2* | *pms1* | *pol30-52* |
|---|---|---|---|---|
| 72 - in | $5x10^{-7}$ | $1x10^{-7}$ | $2x10^{-7}$ | $7x10^{-7}$ |
| 72 - out | $4x10^{-7}$ | $1x10^{-7}$ | $3x10^{-7}$ | $4x10^{-7}$ |
| 78 - in | $5x10^{-6}$ | $2x10^{-7}$ | $4x10^{-6}$ | $7x10^{-6}$ |
| 78 - out | $4x10^{-6}$ | $1x10^{-7}$ | $3x10^{-6}$ | $3x10^{-6}$ |
| | | | | |
| Repetition 2 | *wt* | *msh2* | *pms1* | *pol30-52* |
| 72 - in | $7x10^{-7}$ | $2x10^{-7}$ | $5x10^{-7}$ | $9x10^{-6}$ |
| 72 - out | $2x10^{-7}$ | $1x10^{-7}$ | $3x10^{-7}$ | $2x10^{-6}$ |
| 78 - in | $7x10^{-7}$ | $1x10^{-7}$ | $5x10^{-7}$ | $2x10^{-6}$ |
| 78 - out | $4x10^{-7}$ | $1x10^{-7}$ | $4x10^{-7}$ | $1x10^{-6}$ |
| | | | | |
| Repetition 3 | *wt* | *msh2* | *pms1* | *pol30-52* |
| 72 - in | $3x10^{-7}$ | $2x10^{-7}$ | $1x10^{-7}$ | $9x10^{-7}$ |
| 72 - out | $1x10^{-7}$ | $1x10^{-7}$ | $6x10^{-8}$ | $6x10^{-7}$ |
| 78 - in | $3x10^{-7}$ | $1x10^{-7}$ | $2x10^{-7}$ | $8x10^{-7}$ |
| 78 - out | $1x10^{-7}$ | $1x10^{-7}$ | $8x10^{-8}$ | $8x10^{-7}$ |

The frequency of transmission is the total number of recombinant colonies divided by the limiting parent.

Table 4.4: Mean transmission frequencies for each combination of donor *x* recipient

| | *wt* | *msh2* | *pms1* | *Pol30-52* |
|---|---|---|---|---|
| 72-in | $5x10^{-7}$ | $2x10^{-7}$ | $3x10^{-7}$ | $8x10^{-7}$ |
| 72-out | $2x10^{-7}$ | $1x10^{-7}$ | $2x10^{-7}$ | $9x10^{-7}$ |
| 78-in | $2x10^{-6}$ | $1x10^{-7}$ | $1x10^{-6}$ | $3x10^{-6}$ |
| 78-out | $2x10^{-6}$ | $1x10^{-7}$ | $1x10^{-6}$ | $2x10^{-6}$ |

Table 4.5: Standard deviation for each combination of donor *x* recipient

| | *wt* | *msh2* | *pms1* | *Pol30-52* |
|---|---|---|---|---|
| 72-in | $2x10^{-7}$ | $6x10^{-8}$ | $2x10^{-7}$ | $1x10^{-7}$ |
| 72-out | $2x10^{-7}$ | 0 | $1x10^{-7}$ | $7x10^{-7}$ |
| 78-in | $3x10^{-6}$ | $6x10^{-8}$ | $2x10^{-6}$ | $3x10^{-6}$ |
| 78-out | $2x10^{-6}$ | 0 | $1x10^{-6}$ | $1x10^{-6}$ |

Table 4.6: Standard error (std dev / root 3) for each combination of donor *x* recipient

| | *wt* | *msh2* | *pms1* | *Pol30-52* |
|---|---|---|---|---|
| 72-in | $1x10^{-7}$ | $3x10^{-8}$ | $1x10^{-7}$ | $7x10^{-8}$ |
| 72-out | $9x10^{-8}$ | 0 | $7x10^{-8}$ | $4x10^{-7}$ |
| 78-in | $2x10^{-6}$ | $3x10^{-8}$ | $1x10^{-6}$ | $2x10^{-6}$ |
| 78-out | $1x10^{-6}$ | 0 | $9x10^{-7}$ | $8x10^{-7}$ |

Table 4.7: S17.1(pLH72) and S17.1(pLH 78) *x* MMR mutants

| | | Titres | | | | | |
|---|---|---|---|---|---|---|---|
| | Donors | *wt* | *msh2* | *pms1* | *pol30-52* | Total T | Frequency |
| pLH72 | $3x10^{8}$ | $8x10^{8}$ | $7x10^{8}$ | $5x10^{8}$ | $7x10^{8}$ | 0 | $\leq 3x10^{-9}$ |
| pLH78 | $3x10^{8}$ | $8x10^{8}$ | $7x10^{8}$ | $5x10^{8}$ | $7x10^{8}$ | 0 | $\leq 3x10^{-9}$ |

Values represent the calculated cells/mL of mating mix.

Table 4.8: Mean recombination frequencies $\pm$ standard error for each combination of donors *x* recipient

| | *wt* | *msh2* | *pms1* | *pol30-52* |
|---|---|---|---|---|
| pLH72-inside | $5 \pm 1$ | $2 \pm 0.3$ | $3 \pm 1$ | $8 \pm 0.7$ |
| pLH72-outside | $2 \pm 0.9$ | 1 | $2 \pm 0.7$ | $9 \pm 4$ |
| pLH78-inside | $20 \pm 20$ | $1 \pm 0.3$ | $1 \pm 10$ | $30 \pm 20$ |
| pLH78-outside | $20 \pm 10$ | 1 | $1 \pm 9$ | $20 \pm 8$ |

Transmission Frequencies (x $10^{-7}$) $\pm$ Standard Error ($x10^{-7}$)

The T-tests were used to look for differences between variables (donors and recipients). The T-tests conducted were two-sample equal variance with a two-tailed distribution. Values of a 95% confidence (p-value < 0.05) will be considered statistically different from each other.

Table 4.9: T-test P-values comparing mutants for each combination of donor *x* recipient

| | *wt vs msh2* | *wt vs pms1* | *wt vs pol30-52* |
|---|---|---|---|
| 72 - in | 0.093417 | 0.296657 | 0.083920 |
| 72 - out | 0.205106 | 0.833939 | 0.168959 |
| 78 - in | 0.294062 | 0.761889 | 0.682086 |
| 78 - out | 0.320984 | 0.796222 | 0.898030 |

Table 4.10: T-test P-values comparing plasmids for each combination of donor *x* recipient

| | *wt* | *msh2* | *pms1* | *pol30-52* |
|---|---|---|---|---|
| 72-in vs 72-out | 0.213939 | 0.116117 | 0.785805 | 0.822216 |
| 72-in vs 78-in | 0.378221 | 0.518519 | 0.348072 | 0.296915 |
| 72-in vs 78-out | 0.453562 | 0.116117 | 0.386025 | 0.765446 |
| 72-out vs 78-in | 0.317188 | 0.373901 | 0.331020 | 0.323072 |
| 72-out vs 78-out | 0.364445 | n/a | 0.36197 | 0.438820 |
| 78-in vs 78-out | 0.804378 | 0.373901 | 0.825843 | 0.536054 |

n/a - values were identical

All raw data of the three repetitions were combined into one data set (Table 4.11) and the frequency calculated (Table 4.12). This gives an overview of the assay and gives greater power to the analysis of the data in order to see trends.

Table 4.11: Combining raw data from all three repetitions for each combination of donor *x* recipient. Totals of donors and recipients, and the total recombinant colony number

| | *wt* | *msh2* | *pms1* | *pol30-52* | Donor |
|---|---|---|---|---|---|
| 72 - in | 62 | 5 | 25 | 50 | $1.6x10^{8}$ |
| 72 - out | 37 | 3 | 27 | 43 | $1.7x10^{8}$ |
| 78 - in | 40 | 3 | 31 | 58 | $8.5x10^{7}$ |
| 78 - out | 48 | 3 | 36 | 50 | $7.0x10^{7}$ |
| Recipient | $2.3x10^{8}$ | $4x10^{7}$ | $2.3x10^{8}$ | $9.8x10^{7}$ | |

*Donor and recipient values reported as total number of cells used in all three runs.

Table 4.12: Frequencies of the data reported in table 4.11.

| | *wt* | *msh2* | *pms1* | *pol30-52* |
|---|---|---|---|---|
| 72 - in | $3.9x10^{-7}$ | $1.3x10^{-7}$ | $1.6x10^{-7}$ | $5.1x10^{-7}$ |
| 72 - out | $2.2x10^{-7}$ | $7.5x10^{-8}$ | $1.6x10^{-7}$ | $4.4x10^{-7}$ |
| 78 - in | $4.7x10^{-7}$ | $7.5x10^{-8}$ | $3.6x10^{-7}$ | $6.8x10^{-7}$ |
| 78 - out | $6.9x10^{-7}$ | $7.5x10^{-8}$ | $5.1x10^{-7}$ | $7.1x10^{-7}$ |

4.3.4 Homologous vs Homeologous:

No statistical difference was observed at a 95% confidence interval using MMR- and wt recipients (Table 4.9), or using the plasmid with a homologous vs. homeologous DNA sequence (Table 4.10). Possible explanations for this include 1) reversion of one or more MMR mutants, 2) a mistake in which the 72 and 78 plasmids are actually one or the other only or 3) MMR does not discriminate between the 72 and 78 plasmids when they are introduced using conjugation. The first explanation is considered unlikely because the strains were made by knock-out mutations and were tested for mutator activity using blue white screening (Rodriguez-Beltran, personal communication). The second is also considered unlikely for two reasons 1) as previously stated 72-in, 72-out, 78-in and 78 out were made using *stu*I, *xba*I, *nde*I and *nhe*I, respectively, and pLH72 does not contain the *nhe*I restriction site and pLH78 does not contain *stu*I restriction site which were required for insertion of *oriT* (sequences in Appendix C), and 2) the PCR primers for the *ura3* sequences were specific to pLH72 and pLH78, respectively (see section 2.3.7). The third possibility was tested in a scaled-up experiment using only the wild type as a recipient.

A direct plating conjugation between 72-in and 78-in *x* MPY101 (*wt*) using 10x volume of day culture was conducted (see section 2.2.2). The volumes therefore required for the day cultures were 50 mL for *E. coli* and 500 mL for MPY101. 50 mL of donor and 250 mL of recipient were concentrated into 5 mL of mating mix. The number of recombinant plates was also scaled up by ten, but the same plates were used for this experiment as the previous: SC -Ura -Thr (recombinant), LB + Am (donor) and SC -Thr (recipient). Two repetitions were conducted, the first consisted of a 1:10 ratio and the second consisted of a 1:1 ratio of donor to recipient in the mating mix. The control for this experiment was donors and recipients titre, consistent to that of the mating mix, plated separately on recombinant-selective medium. The negative control previously reported pLH72 and pLH78 have a transmission frequencies less than $\leq 3 \times 10^{-9}$. Recombinant and control plates were incubated for 14 days and then the number of colonies on all 50 plates was counted. There was no growth on the control plates after 14 days and the recombinant numbers for this experiment can be seen in Table 4.13.

NB: p-values were not calculated for this experiment.

Table 4.13: Titres of donors and recipients, and the recombinant colony number per repetition of S.17.1 with 72-in or 78-in *x wt*

| | Donor cells/5 mL | Recipient cells/5 mL | Total Recombinants | Frequency |
|---|---|---|---|---|
| 1:10 | | | | |
| 78-in | $6.0x10^{6}$ | $2.0x10^{8}$ | 250 | $8.3x10^{-6}$ |
| 72-in | $1.0x10^{7}$ | $2.0x10^{8}$ | 183 | $3.7x10^{-6}$ |
| 1:1 | | | | |
| 78-in | $1.0x10^{8}$ | $2.0x10^{8}$ | 145 | $2.9x10^{-7}$ |
| 72-in | $1.0x10^{8}$ | $2.0x10^{8}$ | 174 | $3.5x10^{-7}$ |

Frequency was calculated by multiplying the limiting parent by 5, because the mating mix was made up to 5 mL, and then dividing the number of recombinants by the limiting parent.

The number of recombinant colonies was large enough, using this altered procedure to be certain of the transmission frequencies. However, there were not to conduct statistical analysis on that data within Table 4.13.

### 4.3.5 JB139 *x wt, msh2, pms1* or *pol30-52:*

In order to ascertain if any small observed trends were due to a recombination-specific factor or to growth rate a direct platting conjugation between Yep24 *x* MMR isogenic series was conducted. If no difference were found then the mutation and/or growth rate discrepancies between each individual of the series has no effect on the recombination assay. Therefore, if any difference were observed in the recombination assay, then it is a recombination specific-difference.

A direct plating conjugation procedure between JB139 *x* MPY101, MPY102, MPY103 or MPY104 (*wt, msh2, pms1* or *pol30-52*) was conducted (see section 2.2.2). Three repetitions were performed with the control for this experiment again consisted of the donor and recipients plated onto the transconjugant selective media for each repetition. No colonies were seen on the control plates and the transconjugants were counted after 14 days. The transmission frequencies for each repetition are reported in Table 4.14 and the mean frequency, standard deviation and standard error are reported in Table 4.15 and summarised in Table 4.17. The p-values comparing each member of the isogenic series looking for a difference is reported in Table 4.16. Again the T-test's conducted were two-sample equal variance with a two-tailed distribution. Values of a 95% confidence (p-value < 0.05) will be considered statistically different from each other.

Table 4.14: Titres of donors and recipients, and the recombinant colony number per repetition of JB139 *x wt, msh2, pms1* or *pol30-52*

| Repetition 1 | Cells/mL | Transconjugants | Frequency |
|---|---|---|---|
| *JB139* | $2.0x10^{7}$ | | |
| *wt* | $3.0x10^{8}$ | 7 | $3.5x10^{-7}$ |
| *msh2* | $2.0x10^{8}$ | 2 | $1.0x10^{-7}$ |
| *pms1* | $4.0x10^{8}$ | 2 | $1.0x10^{-7}$ |
| *pol30-52* | $4.0x10^{8}$ | 21 | $1.1x10^{-6}$ |

| Repetition 2 | Cells/mL | Transconjugants | Frequency |
|---|---|---|---|
| *JB139* | $2.0x10^{7}$ | | |
| *wt* | $5.0x10^{8}$ | 32 | $1.6x10^{-6}$ |
| *msh2* | $3.0x10^{8}$ | 56 | $2.8x10^{-6}$ |
| *pms1* | $3.0x10^{8}$ | 191 | $9.6x10^{-6}$ |
| *pol30-52* | $4.0x10^{8}$ | 100 | $5.0x10^{-6}$ |

| Repetition 3 | Cells/mL | Transconjugants | Frequency |
|---|---|---|---|
| *JB139* | $2.0x10^{7}$ | | |
| *wt* | $4.0x10^{8}$ | 30 | $1.5x10^{-6}$ |
| *msh2* | $3.0x10^{8}$ | 100 | $5.0x10^{-6}$ |
| *pms1* | $5.0x10^{8}$ | 16 | $8.0x10^{-7}$ |
| *pol30-52* | $3.0x10^{8}$ | 16 | $8.0x10^{-7}$ |

Table 4.15: The mean, standard deviation and standard error for each combination of donor *x* recipient

| | frequency | | | Mean | St dev | St error |
|---|---|---|---|---|---|---|
| *wt* | $3.5x10^{-7}$ | $1.6x10^{-6}$ | $1.5x10^{-6}$ | $1.2x10^{-6}$ | $6.9x10^{-7}$ | $4.0x10^{-7}$ |
| *msh2* | $1.0x10^{-7}$ | $2.8x10^{-6}$ | $5.0x10^{-6}$ | $2.6x10^{-6}$ | $2.5x10^{-6}$ | $1.4x10^{-6}$ |
| *pms1* | $1.0x10^{-7}$ | $9.6x10^{-6}$ | $8.0x10^{-7}$ | $3.5x10^{-6}$ | $5.3x10^{-6}$ | $3.0x10^{-6}$ |
| *pol30-52* | $1.0x10^{-6}$ | $5.0x10^{-6}$ | $8.0x10^{-7}$ | $2.3x10^{-6}$ | $2.4x10^{-6}$ | $1.4x10^{-6}$ |

St error = St dev / (root 3)

Table 4.16: p-values for JB139 *x wt, msh2, pms1* and *pol30-52*

| | |
|---|---|
| wt vs msh2 | 0.204172 |
| wt vs pms1 | 0.323226 |
| wt vs pol30-52 | 0.301539 |
| msh2 vs pms1 | 0.73232 |
| msh2 vs pol30-52 | 0.809452 |
| pms1 vs pol30-52 | 0.62856 |

Table 4.17: The transmission frequency $\pm$ the standard error for each combination of donor *x* recipient

| | *wt* | *msh2* | *pms1* | *pol30-52* |
|---|---|---|---|---|
| JB139 | $12 \pm 4$ | $25 \pm 14$ | $35 \pm 30$ | $23 \pm 14$ |

Transmission Frequency ($x10^{-7}$) $\pm$ The Standard Error ($x10^{-7}$)

The number of transconjugants was large enough to be confident of the frequency. However, the number of transconjugants did vary by up to an order of magnitude between repetitions.

# Chapter 5: Discussion



*E. coli x S. cerevisiae* conjugation was used to test different conjugation barriers by using different genotypes of *S. cerevisiae* and measuring changes in the DNA transmission frequencies. The results and conclusions for the four hypothesis that have been tested will be discussed in turn, and then brought together to evaluate their impact on conjugation and horizontal gene transfer.

## 5.1 Hypothesis 1: A mutation altering the surface on the recipient *S. cerevisiae* cells can significantly change the transmission frequency

Mutations (ompA) in recipient *E. coli* that have reduced the frequency of plasmid transmission have so far only ever been shown to actually affect donor-recipient contact in liquid mating (Heinemann 1991). If a mutation could be shown to further limit the transmission frequency in *E. coli x S. cerevisiae* crosses, then it could point toward a universal receptor molecule necessary for the initiation of conjugation and/or a less permeable composition of the cell wall or cellular membrane of the recipient. Preliminary work, conducted on an isogenic series of cell wall mutants from INSA, indicated that two of the mutants might possibly be con$^-$ using indirect plating (Merliaud unpublished[1]). The frequency of transmission to recipient strains with different cell wall mutations was measured herein, using the direct plating conjugation procedure to confirm this preliminary finding.

[1] Summer Project Report – Jack Heinemann Lab

Direct plating conjugation between JB139 (YEp24) *x* SY1229 (possible once the selection plates to be used were confirmed) yielded a transmission frequency of 9 x $10^{-7}$ ($\pm$ 1.3 x $10^{-7}$), about 4-times lower than the previously reported frequency of 4 x $10^{-6}$ (Heinemann and Sprague Jr 1991). A four-fold difference in transmission frequency was, however, within the variation previously reported for this procedure (Heinemann and Sprague Jr 1991) and was deemed close enough to continue onto work with the cell wall mutants. The transmission frequencies between JB139 x the isogenic series of cell wall mutants ranged between 3 x $10^{-6}$ and 1.1 x $10^{-6}$ over three replications (Table 3.4). There were no significant differences between the wild type and the mutants as recipients that could be detected using the direct plating conjugation procedure (Table 3.4).

Preliminary work using the indirect plating procedure suggested Kre6 and Mnn9 inhibited conjugation (Merliaud unpublished). Observations made during the course of this work may explain the discrepancy between Merliaud's results and those reported here. Figure 3.2 shows Mnn9 aggregates in solution to a faster and to a greater extent after agitation than every other mutant in the isogenic series. Aggregation was initially ruled out as being capable of limiting *E. coli x S. cerevisiae* conjugation because the solid media prevented aggregation of the recipient in *E. coli x E. coli* conjugation. However, a larger volume of mating mix is plated onto the non-selective plates in *E. coli x S. cerevisiae* conjugation than *E. coli x E. coli* (see section 2.2.2). Larger volumes of solution can be plated and not cause aggregation as long as the plates have been sufficiently dried before hand. If the non-selective plates were not fully dried before use, it is conceivable that the larger volume allowed aggregation of the recipient, which would have caused exclusion of the donors from the recipients, lowering the frequency of transmission.

Kre6, however, did not aggregate to the same extent as Mnn9 but still had the same low recombination frequency for indirect plating (Merliaud unpublished). The Kre6 mutation, however, is known to severely inhibit growth (Jiang et al. 1996) and indirect plating allows 8-12 hours of growth on non-selective medium before cells are replated on transconjugant-selective media (see section 2.2.1). 8-12 hours is enough time for conjugation to occur and several subsequent divisions of the resulting transconjugant *S. cerevisiae* cells on non-selective media. Because Kre6 could not

divide as often as the other mutants on the non-selective media it would have shown a comparatively lower conjugation frequency in indirect plating. Therefore, several individual colonies on transconjugant selective plates may appear from a single conjugation event using indirect plating, and if one recipient strain has a slower growth rate, then the number of recipient exconjugants (as judged by the number of transconjugants) will appear to be less. This systemic error is not possible with direct plating, because each conjugation event leads to a single colony, and even though Kre6 grew more slowly, it showed the same frequency of transmission using this procedure as the rest of the mutants in the series.

Probably the transmission frequency of Kre6 and Mnn9 in indirect plating was influenced by reduced cell-cell contact and growth rate. As such, there is no proven con$^-$ mutation, compared to the wild type, in a recipient for any species once cell-cell contact has been achieved. Cell-cell contact therefore, is one of the strongest limiting steps in conjugation. For instance, cell-cell contact is thought to be controlled in part by the sex pili on the donor cells (Bradley 1980). Sex pili, as previously mentioned, are encoded by *trans-acting* genes of conjugation systems and probably mediate cell-cell contact. Rigid N and P pili have been shown to reduce the frequency of transmission by 1000-fold less in liquid matings compared to solid media (Bradley 1980). These results coincide with the *ompA* mutations in the recipient cells which, because of clumping, also limits transmission 1000-fold in liquid matings compared to solid media (Achtman et al. 1978a; Manning; Achtman 1979; Ou and Yura 1982). By limiting the amount of contact between cells you can severely limit transmission of plasmids. However, this only seems to be the case in liquid.

Importantly, the phenotype caused by ompA, ridgid N and P pili and Mnn9 only seem to limit the amount of cell-cell contact and did not interfere with the process of transfer itself, i.e. transmission once cell-cell contact had been achieved, occurred at the same frequency as the wild type for Mnn9 (Bradley 1980; Manning and Achtman 1979). The difference in wild type transfer between *E. coli x E. coli* compared to *E. coli x S. cerevisiae* is subsequently of interest. The IncF system can conjugate a plasmid at a transmission frequency around 1 in *E. coli x E. coli* crosses (Ferguson et al. 2002) but only at approximately $3x10^{-7}$ in *E. coli x S. cerevisae* crosses (calculated from data in Heinemann 1989). *E. coli* and *S. cerevisiae* have completely different

cellular surfaces which could account for the reduced transmission frequency (Heinemann and Sprague Jr 1989). However, the IncF transfer between *E. coli x S. typhimurium* also has a transmission frequency of approximately $1x10^{-6}$ which is due to internal temperature sensitive factors (Heinemann 1999b). Therefore, the observed reduction in *E. coli x S. cerevisae* could either be due to surface or internal factors.

The surface of *E. coli* consists of an inner and outer membrane sandwiching a thin layer of peptidoglycan (Claverys and Martin 2003). As previously discussed (see section 3.2), the cell surface of *S. cerevisiae* consists of a cellular membrane and an exposed layer of β-1,3-glucan, β-1,6-glucan, chitin and extracellular proteins (Lagroce et al. 2003). *E. coli* and *S. cerevisiae* also have completely different internal cellular organisations. *E. coli* is a prokaryote and as a result everything is contained within one enclosed space. *S. cerevisiae* however, is compartmentalised with DNA partitioned off in the nucleus and the mitochondria. Therefore, this cellular organisation could act as an internal barrier to conjugation. However, *E. coli x S. typhimurium* also has a low transmission frequency due to the *S. typhimurium* restriction modification regime (Heinemann 1999b). But there is no known comparable modification regime in *S. cerevisiae* (Heinemann, personal communication).

No receptor molecule or cellular membrane/wall composition has been shown to be required for conjugation once cell-cell contact is achieved. Conjugation seems quite capable of occurring from bacteria into any other cell once contact has occurred, in almost any environment (Ferguson et al. 2002), although transmission of plasmids seems to vary between species. What accounts for this variation has not yet been found by altering the cellular surface of the recipients as there is no detectable decrease in transmission frequency that can be directly attributed to restricting transfer. If the replication requirements of a plasmid in the recipient are not met, it does not prevent the transmission of DNA. Recombination can occur between plasmids that can not replicate and the chromosome, and as a result conjugation must be one of the most powerful instigators of horizontal gene transfer in the lab and in the environment. In fact, a mutant that increased biofilm formation, which essentially increased cell-cell contact, was recently found in a species that did not conjugate at a high frequency, but did so in this high cell-cell contact mutant (Luo et al. 2005). This

implies that cell-cell contact between donor-recipient is the limiting factor in conjugation and that the cellular surface of the recipient does not have a significant effect if cell-cell contact can be achieved.

Once cell-cell contact has been achieved then the reduced transmission frequency could be due to transportation of plasmid DNA to the nucleus of *S. cerevisiae*. Selby et al. (2006) investigated if 'pilot proteins', transferred into the recipients could be responsible for transportation to the nucleus. They found that the *traI* was localised in the nucleus in both human and *S. cerevisiae* cells, but the pilot protein for a different conjugation system IncQ (MobA) was not localised in the nucleus. This indicates that DNA makes its way to the nucleus un-aided by proteins transported across with it. As a result, it is not know how DNA navigates to the nucleus, but once there recombination can occur. Recombination-dependent conjugation is important because small incremental changes to DNA sequence through recombination are important in prokaryotic evolution, which is summed up nicely by the claim by Denamur et al. (2000) that any mutation in a bacterial genome is 50 times more likely to come from recombination than by any other means. The same pattern of evolution in eukaryotes is however, never accredited to horizontal gene transfer but instead credited to an increased mutation rate, termed punctuated evolution (Denamur and Matic 2006; Pagel et al. 2006).

The Heinemann model can explain punctuated evolution in prokaryotes through environmental stress preferentially selecting for mutator phenotypes with an increased ability to alter their DNA sequence through polymerase errors or increased recombination (Heinemann and Billington 2004). However, an increased recombination mutant has not yet been observed for intermolecular recombination in *S. cerevisiae* (Porter et al. 1996; Buchanan 2002). If there is no mutant that can increase recombination between intermolecular substrates, then the previous reported findings that sequence similarity has little intermolecular recombination tells us that, the amount of DNA a eukaryotic genome is exposed to is the limiting factor to recombination and possibly punctuated evolution.

5.2 Hypothesis 2: The frequency of recombination will be higher when homologous DNA is delivered to *S. cerevisiae* by conjugation, as compared to delivering homeologous DNA.

As previously mentioned, the frequency of recombination was found to increase exponentially with increasing sequence similarity in Bacillus, *E. coli* and in *S. pneumoniae* (Majewski and Cohan 1999; Rayssiguier et al. 1989; Majewski et al. 2000, respectively). Distinguishing between DNA molecules based on differences in sequence similarity was subsequently considered the mechanism of maintaining species isolation in prokaryotes (Majewski and Cohan 1999). In *S. cerevisiae,* sequence similarity has also had a significant effect on the frequency of recombination between sequences within the same chromosome (intrachromosomal), especially in meiotic cells (Hoffman 1991; Selva et al. 1995). These results lead to claims that sequence similarity could also be the mechanism of maintaining species isolation in eukaryotes (Hunter et al. 1996). Intermolecular work in mitotic cells, however, showed that recombination between a plasmid and a chromosome had little (within the same magnitude) if any effect on the frequency of recombination (Porter et al. 1996 and Buchanan 2002). And, recombination between dissimilar (52%) plasmids occurs in yeast (Mezard et al. 1992). Extrapolation of these results to this experiment indicates that plasmids delivered by conjugation into mitotic cells should recombine close to the same frequency (within the same order of magnitude) in the wild type independent of sequence similarity. Why sequence similarity affects intrachromosomal recombination in meiotic *S. cerevisiae* and not intermolecular recombination in mitotic cells is a curious observation that requires further investigation due to its important implications for eukaryotic evolution.

Therefore, in order to be confident with the results of sequence similarity in intermolecular recombination, two donor plasmids that contain sequences that have previously shown a marked difference in intrachromosomal recombination (Hoffman 1991), were modified for use in *E. coli x S. cerevisiae* conjugation. The plasmids were modified through a series of optimised steps (Figure 4.7). One plasmid was used to optimize this procedure (Appendix D) and then the final three plasmids were created once the first one was completed. The four constructed plasmids were shown to transfer at frequencies consistent with RP4 (another IncP plasmid) in *E. coli x E. coli*

indirect plating (Table 4.1) (Ferguson et al. 2002). The original S17.1 strain containing the original pLH plasmids, S17.1 (with no plasmids) *x* DE1661, and equivalent titres to the mating mix of the donors and recipient were each separately plated on the transconjugant media as controls for this experiment. These were conducted to gauge the frequency of transmission for the original plasmids and the integrated RK2, to check the plates and genotypes of the parents. No colonies were seen on such plates, and the resulting transmission frequencies are reported (Table 4.1). The results show that the pLH78 derived plasmids have a slightly higher transmission frequency than those derived by pLH72, but the frequency for all of the plasmids was well within the normal range for IncP plasmids (Ferguson et al. 2002). The donor S17.1 with the modified pLH plasmids could now be used to test the effect of sequence similarity on the frequency of recombination utilising direct plate conjugation.

All of the four strains of donors containing the plasmids created herein (72-in, 72-out, 78-in and 78-out) were used in a conjugation with all four *S. cerevisiae* (*ura3-kpn*I inserted) using the direct plating procedure. The transmission frequencies (Table 4.8) show that the mean frequencies of transmission are all within an order of magnitude of each other. The standard deviation and p-values (see Table 4.5 and 4.10, respectively) for each mean frequency indicates that there was no significant difference (p-value < 0.05) between the frequencies of recombination between homologous (72-in and 72-out) and homeologous (78-in and 78-out) DNA sequences in any mutant.

The difference observed for these sequences in intrachromosomal recombination was 60-fold ($6.7x10^{-7}$ and $1.16x10^{-8}$ for the homologous and homeologous sequences, respectively) (Hoffman 1991). The difference observed for these sequences in the recombination-dependent-conjugation assay was 4-fold ($5x10^{-7}$ and $2x10^{-6}$ for the homologous and homeologous sequence respectively) (Table 4.8) Note: that this is the data for 72-in and 78-in. The homeologous (78-in and 78-out) sequences recombined at a frequency 5-times higher than the homologous (72-in and 72-out) DNA, contrary to previous reports (Porter et al. 1996; Selva et al. 1995). However, as mentioned above there is no statistical difference between these values (Table 4.10) and whereas

Buchanan (2002) did find a statistical difference between homologous and homeologous DNA substrates in his transformation assay.

The transformation experiment conducted by Buchanan (2002) standardised the amount of DNA and the number of cells for each repetition of the assay. Because of this standardisation, Buchanan (2002) was able to find a statistical difference between the homologous substrate and the homeologous substrate even though the recombination frequencies were within the same order of magnitude. However, the DNA was quantified using a spectrophotometer that only read at 260nm. Measurements at only this wavelength are prone to overestimation due to contamination. To detect contamination and be certain of the actual DNA concentration, a measurement should have been made measuring the ratio of 260nm/230nm. The concentration of the plasmid isolate solutions used in Buchanan (2002) could therefore, have differed by an order of magnitude due to different amounts of contamination between stock plasmid preps. Because of the standardisation of the procedure, the same amount of DNA and number cells were used in each repetition of the assay. The observed statistical relevance could, therefore, have been a bi-product of an error occurring before standardisation of variables.

In order to understand the lack of significant difference in the frequencies of recombination the raw data was re-examined. Examination of the raw data (see Table 4.2) shows that the number of recombinants between each repetition varied greatly for each combination of variables (donor and recipient). For example, in the wild type, the number of recombinants reported ranged between 48-7, 32-2, 26-7 and 40-4 for 72-in, 72-out, 78-in and 78-out respectively (Table 5.1). Surprisingly for 78-out, in repetitions 1 and 2, the number of recombinant colonies drop by an order of magnitude when recombination in the wild type recipient is measured (Table 5.1), even though the same titres of donors and recipients were used in both replications (Table 4.2). The resulting frequency (Table 4.3) subsequently varies by an order of magnitude for repetition 1 and 2. The first repetition for both of the pLH78-derived plasmids has a significantly higher transmission frequency than the other two repetitions. This both results in a higher mean transmission frequency and a higher standard deviation. The transmission frequencies of the homologous DNA (72-in and

72-out) did not vary as much as the homeologous (78-in and 78-out) (Table 4.3), even though they experienced the same variation in transconjugant numbers between repetitions. But as mentioned previously, the homologous DNA had on average a lower frequency of recombination. Homeologous DNA therefore, has on average a greater recombination frequency (Table 4.8), but also has a greater variation that either caused or obscured any statistical difference (Table 4.10)

Table 5.1: Number of *wt* recombinant colonies observed in each repetition of S.17.1 with 72-in, 72-out, 78-in or 78-out *x wt, msh2, pms1* or *Pol30-52*

| | repetition 1 | repetition 2 | repetition 3 | Range |
|---|---|---|---|---|
| 72-in | 48 | 7 | 7 | 48 to 7 |
| 72-out | 32 | 2 | 3 | 32 to 2 |
| 78-in | 26 | 7 | 7 | 26 to 7 |
| 78-out | 40 | 4 | 4 | 40 to 4 |

Greater variation of the pLH78-derived plasmids may have obscured any statistical difference. However, removal of the first repetition results in both the pLH78 and the pLH72-derived plasmids having the same recombination frequency in the wild type (Table 5.2). But what may account for this statistical difference is hidden within the first repetition of this experiment, which contained the largest number of recombinants for the wild type (Chapter 4: Table 4.2) and also the biggest difference in the transmission frequency between homologous and homeologous sequences (Chapter 4: Table 4.3). The donor to recipient ratio, however, differed in this repetition by approximately 1:1 for the homologous (72-in and 72-out) sequence, and approximately 1:10 (donors:recipients) for the homeologous (78-in and 78-out) sequence (Chapter 4: Table 4.2). In the second repetition of this experiment all of the wild type combinations were at 1:10 (donors:recipients) and subsequently all of the transmission frequencies were relative to each other (Chapter 4: Table 4.3). The donor to recipient, ratio therefore, seems to have a large effect on the frequency of recombination.

Table 5.2: Mean frequency (minus first repetition)

| | *wt* |
|---|---|
| 72-in | $4.7 \times 10^{-7}$ |
| 72-out | $1.5 \times 10^{-7}$ |
| 78-in | $4.7 \times 10^{-7}$ |
| 78-out | $2.7 \times 10^{-7}$ |

In order to test if the donor to recipient ratio changes the frequency of recombination and to increase the number of recombinants, donors containing 72-in and 78-in were crossed with the wild type. For this assay, the volume of donor and recipient day cultures was increased 10-fold. Two repetitions were conducted, the first with a donor to recipient ratio of 1:10 and the second with a ratio of 1:1. The resulting frequencies (Table 4.13) show that at 1:10 the frequencies of recombination are a full order of magnitude above the 1:1 even though the number of total recombinants remains very similar. This is consistent with the observation mentioned above, that the first repetition differed because of the donor to recipient ratio, even though they had similar transconjugant numbers (Table 4.2). This assay also shows that in the 1:1 repetition the transmission frequency of the homologous plasmid is higher than the homeologous and in the 1:10 repetition the reverse is true (Table 4.2). This successfully shows that the donor to recipient ratio has a greater effect on the frequency of recombination than the sequence similarity and that the same donor-recipient ratio is required to compare transmission frequencies.

The effect of sequence similarity on recombination is therefore, obscured by the variation within this assay, and that variation is caused by the donor to recipient ratio. Heinemann and Sprague Jr (1991) optimised the donor to recipient ratio in order to obtain the highest frequency of transmission per limiting parent (i.e. donor or recipient). This work shows that there is an optimal donor to recipient ratio for conjugation-dependent on recombination as well. Heinemann and Sprague Jr (1991) attributed the difference in transmission frequency to *E. coli* and *S. cerevisiae* having different environmental preferences and the possibility that *E. coli* is in some way inhibitory to *S. cerevisiae* growth. Therefore, the fewer *E. coli* the easier it is for yeast to recover from conjugative DNA transfer and express a new gene. This could possibly be extrapolated to this data herein indicating that the same is true for recombination-dependent-conjugation events. For this to be true, the same proportion of plasmids must be successfully recombining in the recipients independent of sequence similarity. If recombination occurs independent of sequence similarity then sequences delivered by conjugation can recombine with the chromosome at the same frequency whether homologous or homeologous. This is in stark contrast to *E. coli* that shows an exponential relationship for sequence similarity and recombination, with sequences delivered by conjugation (Majewski et al. 2000).

Recombination in *S. cerevisiae*, therefore, has different recombination frequencies relative to the substrate and growth phase. The homologous and homeologous DNA used herein has previously been shown to have a different recombination frequency in meiotic and mitotic intrachromosomal recombination (Hoffman 1999). Intramolecular recombination typically had a higher recombination frequency in meiotic cells than mitotic cells (Hoffman 1999; Selva et al. 1995). The homologous and homeologous DNA used herein did not show any difference (Table 4.10) between recombination frequencies in mitotic intermolecular recombination. Intermolecular recombination has had little difference between homologous and homeologous recombination (Buchanan 2002; Porter et al. 1996).

This suggests two things. First, growth phase and the affect of sequence similarity on recombination are linked (a mechanism may be preventing recombination in mitotic growth phases that is relaxed in meiosis). Second, intermolecular recombination is only marginally dependent (if at all) on sequence similarity.

Intermolecular recombination between homologous and homeologous substrates have so far all been within an order of magnitude (Buchanan 2002, Porter et al. 1006 and this work). Sequence similarity, therefore, acts as a barrier to recombination for intrachromosomal sequences but not for intermolecular sequence recombination. Sequence similarity also inhibits intrachromosomal sequence recombination to a greater extent in meiotic cells than mitotic. The relevance of this is that there seems to be a general barrier to intermolecular recombination in *S. cerevisiae* cells. This barrier may also lower homologous and homeologous DNA recombination in mitotic cells but not in the meiotic phase of growth. This general barrier to recombination inhibits recombination to a low level for all substrates in mitotic growth and therefore, recombination frequency is affected by the amount of DNA but not sequence similarity.

An experiment that introduced two plasmids into a *S. cerevisiae* indicated that recombination can occur between vastly different (52%) plasmid sequences (Mezard et al. 1992). If the same is shown to be true in all eukaryotes then chromosomal

exposure to DNA will alter the sequence of the chromosome at the same frequency independent of the sequence similarity, but dependent on the amount.

5.3 Hypothesis 3: The frequencies of recombination between a chromosome and either a homologous or homeologous DNA sequence on a plasmid will differ between wild type and MMR- strains.

In prokaryotic recombination, the sequence similarity required between two DNA molecules to successfully recombine is increased by MMR. Recombination between diverged sequences increases by up to 1,000 fold in MMR mutants (Funchain et al. 2001; Heinemann and Billington 2004; Zahrt and Maloy 1997). Selection for recombinants with homeologous DNA therefore, also preferentially selects for MMR deficient individuals (Funchain et al. 2001). Sequencing of *E. coli* MMR genes has shown that they have been widely transferred among prokaryotes and as a result it is estimated that any mutation is 50 times more like to come from recombination than by any other means (Denamur et al. 2000). *A* increased frequency of recombination between homeologous sequences during intrachromosomal recombination in different MMR mutants of *S. cerevisiae* has been shown (Selva et al. 1995). As such Hunter et al. (1996) proposed that the MMR proteins contributed towards the establishment of post-zygotic species barriers. However, Porter et al. (1996) and Buchanan (2002) did not find either the frequency of homologous and homeologous DNA recombination was significantly altered in MMR mutants.

This recombination-dependent-conjugation assay involved the transfer of either homologous or homeologous DNA into various MMR$^-$ *S. cerevisiae* through conjugation. Because transformation-dependent recombination with *msh2* and *pms1* has already been conducted by Porter et al. (1996) and verified by Buchanan (2002), any variation found between the results obtained with this assay and the results previously reported will indicate that conjugatively delivered DNA may have a physically different structure to plasmid DNA introduced by transformation. At a 95% confidence level there is no statistical difference between mutants and the wild type with either of the DNA substrates (Table 4.9), which could possibly be the variance getting swamped by small changes in the donor to recipient ratio. However, at a 90% confidence interval both *msh2* and *Pol30-52* have a statistical difference compared to

the *wt* with 72-in (Table 4.9). The mean transformation frequencies (Table 4.8) shows that *msh2* decreases and *Pol30-52* increases the frequency of recombination compared to the wild type for every plasmid. However, all of the mean transmission frequencies (Table 4.8) are well within an order of magnitude of each other, which was shown above to be the natural variation of this assay taking into account the donor to recipient ratio (Table 4.13).

In order to discern if there is a difference between *msh2*, *Pol30-52,* and the *wt,* all of the three repetitions of this assay were added together into one data set (Table 4.11). The total donors and recipients as well as the recombinant numbers have lower recombination frequencies (Table 4.12) compared to the mean transmission frequencies (Table 4.8). All of the frequencies are still within an order of magnitude of each other. However, the number of transconjugants colonies formed by *msh2* is far fewer than the other members of the series. Fewer transconjugant colonies may indicate that it has trouble recombining, although *msh2* still remains within an order of magnitude to the wild type because it also has a low number of recipient cells (Table 11). It must therefore, be considered that the slower growth rate of the *msh2* mutation could have prevented the growth of recombinants and the reduced transconjugant colony number was not anything to do with the recombination frequency. Growth rate of *S. cerevisiae* was observed to not affect *E. coli x S. cerevisiae* conjugation in the cell surface experiments. Therefore, in order to see if *msh2* specifically affected the recombination frequency or if the reduced transconjugant colony formation was a result of the growth rate, a conjugation was performed with the isogenic series and JB139. If transmission of YEp24 occurs equally well in all members of the isogenic series then transfer is not inhibited by growth rate and recombination must have been specifically affected.

Transmission of YEp24 into the isogenic series by conjugation with E. coli showed that YEp24 had transmission frequencies within the same order of magnitude into all the members of the isogenic series (Table 4.17). T-tests conducted on the raw data (Table 4.14) showed that there was no statistical difference (p-values $<0.05$) between any of the mutants (Table 4.16). No statistical difference indicates that a reduced growth rate did not prevent the formation of transconjugants and that if there is a factor affecting *msh2* it must be recombination-specific. Msh2p in *S. cerevisiae* is

required for nonhomologous tail removal (Nicholsen et al. 2006), which as previously reported, SSA is dependent on (Goldfarb and Alani 2004). SSA represents a major pathway for the repair between repeated sequences in the same chromosome, which results in a deletion of DNA between the repeats (Goldfarb and Alani 2004). SSA can also occur between chromosomes, resulting in deletion of one or two sections of DNA (Paques and Haber 1999). Subsequently, a recent paper conducting interchromosomal recombination found that both Msh2p and Msh3p reduced the frequency of recombination compared to the wild type (Nicholson et al. 2006).

Porter et al (1996) utilized an *msh2* mutation in their transformation assay and found no inhibitory affect for *msh2*. This indicates that the DNA substrate in their assay recombined using a different recombination pathway compared to the DNA substrate in this recombination-dependent-conjugation assay (this work). In Porter et al. (1996) *S. cerevisiae* was transformed with a double-stranded circular single copy plasmid, and in this assay *E. coli x S. cerevisiae* transferred a double-stranded circular non replicating plasmid by conjugation. The DNA in Porter et al. (1996) probably entered the nucleus as a double-stranded closed circular plasmid and then recombined with the chromosome which as previously mentioned, would probably have been induced by a double-stranded break (Paques and Haber 1999). It is unknown where and when plasmids transferred by conjugation are converted back into a double-stranded molecule in eukaryotes and this could be seen as corroborating evidence that a transferred plasmid is single-stranded when it enters the nucleus. If the post conjugation single stranded DNA had been converted back into a double-stranded closed circular plasmid before it entered the nucleus then the same frequency of recombination in *msh2* should have been observed. Because a double-stranded break is normally required to initiate recombination, the transformed DNA in Porter et al. (1996) was probably converted into a linear plasmid just before recombination. So if the conjugatively transferred plasmid was only converted into a double-stranded molecule, the *oriT* ends would most likely be the ends of the linear DNA and would be the same substrate initiating recombination. Therefore, the most probable explanation for the difference in the *msh2* mutant between Porter et al. (1996) and this work is that the DNA in this assay is single stranded when it enters the nucleus.

Different recombination pathway have been previously associated with double-stranded DNA and single-stranded DNA. Singh et al. (1982) found that "single-stranded DNA molecules can participate in a recombination pathway that trims one or both DNA recombination substrates, a pathway not detected, at least at the same frequency, when transforming with double-stranded DNA molecules". This was confirmed by Simon and Moore (1987) who concluded that "single-stranded DNA may participate directly in recombination with the chromosome". Both of which, are characteristic of the SSA recombination pathway described above. However, because recombination still occurred in *msh2* single-stranded DNA only preferentially go through a *msh2* dependant recombination pathway. Alternatively the single stranded plasmid could be replicated and reform a double-stranded molecule before it recombined with the chromosome. The pathway that double-stranded plasmids mainly go through must be a Msh2p independent pathway.

Transfer of YEp24 via conjugation into the isogenic series tells us nothing about *Pol30-52*, because it has the same recombination frequency compared to the wild type (Table 4.17). The combined data tables indicate that *Pol30-52* also has no difference compared to the wild type in the recombination-dependent conjugation assay (Table 4.11 and 4.12). However, the raw data (Table 4.2) indicates that this mutant always had more transconjugants than the wild type, and a slightly higher transmission frequency (Table 4.3). The trend observed in this work has suggested that it may have a slightly increased the frequency of recombination. Unfortunately, the only way to be certain of the trends for *msh2* and *Pol30-52* described herein is by getting more recombinants. Therefore 10x experiments with all four members of the *S. cerevisiae* series and 72-in will have to be conducted at a later date (because this is a very large scale experiment). The preliminary results are interesting in that the *msh2* and *Pol30-52* had counter effects to each other and *msh2* tells us that conjugated DNA probably engages in a SSA recombination pathway, but it also indicates that conjugated plasmids travel to the nucleus in a single stranded form, before being converted into a double-stranded molecule.

5.4 Hypothesis 4: The position of the *oriT* will have an effect on the frequency of recombination.

Previous work has found that the proximity of a DSB can affect the frequency of recombination in intrachromosomal recombination (Surtees et al. 2004). It is possible that following replication of the transferred plasmid back into a double-stranded molecule that the *oriT* would become the DSB and initiate recombination (discussed above). Locating the DSB close to the *ura3* and 2kb away from the *ura3* should show a difference in recombination frequency if this were the case. The other possibility is that because the *oriT* is highly dissimilar to *ura3*, the close proximity of highly dissimilar sequences to the gene of interest may negatively affect the frequency of recombination. There is however, no statistical difference (p-value > 0.05) when *oriT* is located within or distal to the *ura3* allele (Table 4.10). Although in each repetition of the assay (Table 4.2) *oriT* inside the *ura3* recombined slightly more that outside, which is reflected in the mean frequencies (Table 4.8). However, combining all of the raw data into one experiment, surprisingly had the opposite effect (Table 4.12). However, the affect doest not seem to be as large an effect as the donor to recipient ratio. The trend, as a result, does not exist. This tells us that in horizontal gene transfer it does not matter where on a plasmid a gene is positioned, the likelihood that two genes will recombine and be inherited are the same regardless of its physical location.

## 5.5 Barriers to transmission

Conjugation has been observed between bacteria *x* bacteria, fungi (Heinemann and Sprague Jr 1989), plant (Christie et al. 2005; Marton et al. 1994), hamster (Waters 2001) and human (Kunik et al. 2001) cells. Conjugation is thought to help induce biofilms (Sorensen et al. 2005) and the transmission of genes from ‘dead’ to living cells (Redfield 1988). Bacteria have been shown to invade human cells and conjugate with other bacteria, resulting in both plasmid (Ferguson et al. 2002) and even chromosome transfers (Kurenbach, Deng and Heinemann unpublished). Conjugation as a result has been instrumental in transferring antibiotic resistance among prokaryotic species (Bradford 2001). However, conjugation can also allow DNA transfer back from the recipient (Heinemann et al. 1996) and the transportation of proteins into the recipients (Heinemann 1999a). Subsequently, conjugation is one of the most versatile and significant forms of DNA transfer inside and outside the Laboratory. But, how and why conjugation can occur into such a wide range of species, and out inability to find a con$^-$ mutant is probably the most fundamental to our understanding of the system itself.

Transfer into *S. cerevisiae* was not limited by any of the members of the cell wall isogenic series. The lack of recombination of the *msh2* mutant in the assay conducted herein compared to Porter et al. (1996) indicates that postconjugation plasmid DNA within the recipient is single-stranded when recombining with the chromosome. We also know because of using different sequences and different insertion sites for *oriT* that once transferred into a *S. cerevisiae* cell, the sequence similarity compared to the chromosome and the location on the chromosome has little or no effect on recombination. This suggests that there is no barrier to transmission of DNA once cell-cell contact has been achieved.

As previously mentioned, prokaryotes distinguish between homologous and homeologous sequences in recombination and further restrict the required sequence similarity by utilizing MMR (Majewski and Cohan 1999). This occurs to a similar extent in meiotic and to a lesser extent in mitotic cells between intrachromosomal sequences in *S. cerevisiae* (Selva et al. 1995). MMR is seen as enforcing species isolation by sequence similarity in prokaryotes (Majewski and Cohan 1999), but this does not seem to apply in this model eukaryote. DNA delivered by both transformation (Porter et al. 1996; Buchanan 2002) and conjugation (this work) have shown that sequence similarity has no clear relationship with recombination frequency and MMR does not further restrict constraints on sequence similarity. Sequence similarity and MMR in *S. cerevisiae* therefore, act on different substrates differently.

Interestingly, plasmid DNA reisolated from simian cells indicated that there was a high mutation rate and some of the host chromosome had inserted into the plasmid (Carlos et al. 1983). If the results from the work conducted herein are representative of eukaryotes in general, then recombination with the chromosome would have occurred independent of sequence similarity at the same low frequency. The same observation has been seen in *S. cerevisiae* recipient plasmids after reisolation found that the restriction map of plasmids had been altered (Heinemann personal communication).

If recombination occurs between the plasmid and the chromosome at a low level then eukaryotes may follow the Heinemann model. Two illustrative examples are provided by studies on *Arabidopsis thaliana* (Kovalchuk et al. 2000) and chemotherapy drug resistance (Hoeijmakers 2001 and Pors et al. 2005). Kovalchuk et al. (2000) reported different frequencies of increased mutation rated between different lines of transgenic *A. thaliana.* The observed variation in mutation levels could be due to different mutation rates in different parts of the chromosome i.e. the gene was inserted into different parts of the chromosome (as was argued by the authors). Localisation of the inserted genes was not however, provided for any of the plants in this paper to confirm this hypothesis. Another explanation for the variable mutation rates in these plants is that introduction and selection for foreign DNA selected for mutators with and increased recombination rate. These mutators that have a higher rate of recombination than the wild type have not yet been found in *S. cerevisiae*. However, if they exist, they are more likely to participate in and subsequently be selected for in this type of reaction. The increased proportion of mutators within the population of *A. thaliana* would, as a result, increase the mutation rate.

## 5.6 Cancer

Chemotherapy agents target tumour cells using the characteristics that differentiate them from normal somatic cells. One common mechanism of action for chemotherapeutic agents is to damage the DNA and thereby block cell division, induce apoptosis, and/or increased the mutation rate within the tumour cells (Hoeijmakers 2001). Unfortunately this leads to side effects that affect other fast replicating cells naturally present in our bodies, i.e. hair follicles, stomach and gastrointestinal linings. What is of interest to this work, is that the DNA of these faster replicating cells is directly targeted by some chemotherapies, i.e. with platinating and metabolites (Hoeijmakers 2001; Pors et al 2005). Through targeting the DNA, MMR deficiency is selected for in tumou cells. MMR deficiencies could be selected for because once MMR detects mismatches MMR can (1) not repair the sequence, (2) initiate cell-cycle arrest and (Hoeijmakers 2001), (3) initiate apoptosis (Hoeijmakers 2001).

Therefore, cancer cells treated with these agents that abandon a functional MMR are able to continue replicating. Note here that the only tumour cells to survive the DNA

damage are those that do not enter apoptosis, forgoing repairing of the damaged DNA and continue replicating, all of which is controlled by Msh2p in prokaryotes and eukaryotes (Jirncy 2006). The Heinemann model of increasing mutator population due to environmental stress seems to therefore, hold true for tumors. The work conducted herein, however indicates that recombination happens independent of sequence similarity in *S. cerevisiae.* Holmgren et al (1999) point out there "exists stages during tumor development in which there is a high turnover of genetic material that is fragmented during apoptosis and subsequently taken up by neighbouring cells". Intermolecular recombination is independent of sequence similarity, and cancer cells will subsequently be immersed in DNA that can enable adaptation to chemotherapies.

However, the cancer cells themselves have been shown to become almost parasitic (Murgia 2006; von Holt and Ostrander 2006), infecting different breeds of dogs. So cancers can spread through cellular mechanisms and possibly through horizontal transfer of DNA.

5.7 Phylogenetics

Keeling et al. (2005) commented that the "debate over the role of Lateral Gene Transfer in eukaryotes has lagged behind the prokaryotic debate". This is possibly because inheritance in eukaryotic genomes is limited to those events that affect the gametes or zygotic cells (germline) and selection as a result for correctly functional cells can be high in eukaryotes (Pizzari and Birkhead 2002), But this only selects against traits that effect the gametes or zyghotic cells. A startling case that clouds the issues is Chagas disease. Chagas disease is caused by *Trypanosoma cruzi* and Nitz et al. (2004) demonstrated that it is due to transfer of mitochondrial DNA (kDNA) into the host cells and recombination with the host's mitochondrial genome. They also found that after infection the offspring of individuals were infected even after only short-term infections (Nitz et al. 2004). However, Nitz et al. (2004) has been subject to the first forced post publication retraction in, the Journal, Cell's history (Marcus 2005) and it is unclear what data within the paper is valid and what is not.

A basic misunderstanding of HGT that is very commonly held ignores small alterations to a genome even through it has previously been shown to be fundamentally important to prokaryotic (Majewski et al. 2000) and eukaryotic

(herein) evolution. The transfer of large amounts of DNA encoding fully functional genes is not necessary to have an affect on evolution. Lawrence and Ochman (2002) state that "short sequences (<500bp) often appear atypical for stochastic reasons and might be misidentified as having been transferred", to which immediately limits horizontal transfer to DNA of greater than 500bp in length. The length of DNA that was required to be altered in the conjugation/recombination assay conducted herein was approximately 5bp to reactivate the chromosomal *ura3-kpnI* gene. The *oriT* fragment was 285bp and would therefore also not be considered capable of misidentification.

## 5.8 Future work

Areas of study that could be conducted

- Sourcing an increased recombination mutant in *S. cerevisiae* by developing an assay that can mass screen for a recombination mutant.
- Conduct three repetitions of a 10x day culture experiment on all members of the isogenic series with 72-in to confirm trends seen herein.
- Sequence *URA3* from frozen down transconjugants to find out what type of recombination events occurred.
- Conduct a transformation assay of all the members of the isogenic series with 72-in to compare to 10x experiments.
- Create *msh2* and *msh6* members of isogenic series and conduct assay to confirm predicted decrease in recombination in *msh3* (Msh2p-Msh3p are involved in non-homologous tail removal)

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