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Characterization of a novel class of mutation (Short Patch Illegitimate Recombination) in *Acinetobacter baylyi*

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Abstract

In prokaryotes, genetic variability contributes to the adaptation to novel environmental conditions, to resistance against threats such as antimicrobial agents and viruses, and ultimately to bacterial evolution through selection and drift. Genetic variability is mainly generated through mutations, e.g. point mutations, deletions, insertions, inversions, and duplications/amplifications. In addition, horizontal gene transfer (HGT) can contribute to variability by enabling a cell to acquire genetic material from closely (or more rarely, from distantly) related organisms. DNA taken up by HGT can be recombined with the chromosome of the recipient cell by either homologous recombination or illegitimate recombination. In this study a novel type of illegitimate recombination where small patches of DNA are recombined at microhomologous DNA segments, termed short patch illegitimate recombination (SPIR) is described in *Acinetobacter baylyi*. The results suggests that SPIR events typically lead to a substitution of 2-8 base pairs alone or together with a 3-9 base pair deletion, or a larger deletion up to 168 base pairs. It was also shown that SPIR occurs both in transformable and non-transformable strains, and that SPIR frequency is decreased by presence of the 3'-5' single strand specific exonuclease ExoX. It is conceivable that SPIR enables greater changes in a genome over the course of a single generation than earlier described mutation mechanisms, and can thus play a role in adaptive evolution.

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Abbreviations

Amp^r	Ampicillin resistant
bp	Base pairs
Cm^r	Chloramphenicol resistant
dH_2O	Distilled water
DIR	Double Illegitimate Recombination
DNA	Deoxyribonucleotide acid
HFDIR	Homology Facilitated Double Illegitimate Recombination
HFIR	Homology Facilitated Illegitimate Recombination
HGT	Horizontal gene transfer
IR	Illegitimate Recombination
Kan^r	Kanamycin resistant
LB	Luria-Bertani medium
MGE	Mobile genetic element
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Rif^r	Rifampicin resistant
rpm	Rounds per minute
SNP	Single nucleotide polymorphism
SPIR	Short Patch Illegitimate Recombination
Suc^s	Sucrose sensitive
TAE	Tris-acetate-EDTA
UV	Ultra-violet irradiation

1. Introduction

1.1. Preface

Bacterial genomes are shaped by natural selection, commonly regarded as the crux of evolution. In bacteria, as well as other organisms, natural selection acts on pre-existing inheritable genetic diversity and contributes to adaptation to environmental conditions such as resistance to environmental stressors (e.g. antibiotic resistance and bacteriophage). The concept of natural selection was exemplified by Charles Darwin and his "Galapagos finches" where he correctly assumed that pre-existing diversity in finch beaks were selected for in the existing environmental conditions (DARWIN 1859).

1.2. Causes of genetic diversity in bacteria

Diversity is mainly created through rare and randomly occurring mutations, here broadly defined as any inheritable change in DNA. Bacteria are generally useful for studying mutation since they replicate fast and generate large populations quickly. The effects of mutations on a cell can range from beneficial to neutral to deleterious. Mutations can either be induced by a mutagen such as UV radiation and various chemicals, or occur spontaneously through point mutations (Figure 1), deletions (BIERNE *et al.* 1997; GORE *et al.* 2006; IKEDA *et al.* 2004), inversions (CRAIG 1985), insertions (MAHILLON and CHANDLER 1998) and gene amplifications (ANDERSSON and HUGHES 2009). Bacteria can also engage in horizontal gene transfer mainly through the processes of transduction (MAJEWSKI 2001), conjugation (CHEN *et al.* 2005; FROST *et al.* 2005), and natural transformation (LORENZ and WACKERNAGEL 1994), and to a lesser extent through nanotubes (DUBEY and BEN-YEHUDA 2011), and outer-membrane-vesicle mediated DNA transfer (YARON *et al.* 2000). DNA acquired horizontally can sometimes lead to genomic rearrangements through recombination. The known recombination mechanisms are homologous recombination and illegitimate recombination (IR) (KOWALCZYKOWSKI *et al.* 1994; PERSKY and LOVETT 2008).

1.2.1. Point mutations

Point mutations are typically substitutions of single nucleotides, but less frequent deletions and insertions of single nucleotides also occur. These usually happen due to errors during

replication, and can be reversed through a second point mutation. The relevance of a point mutation depends on its location. If a point mutation causes the addition or loss of a nucleotide within a gene, a frameshift occurs, resulting in an altered gene product. Point mutations can be silent, lead to altered amino acid sequences through a missense mutation, or stop translation entirely through a nonsense mutation (Figure 1). DNA polymerases typically contain proofreading functions that prevent point mutations. Such functions ensure genomic stability. To avoid continuous damage through random deleterious mutations, bacteria have evolved mechanisms that avoid point mutations, such as DNA mismatch repair and DNA proofreading polymerases. These functions ensure metabolic stability, but also come with a high metabolic cost. Evidence suggest that DNA based organisms have reached a balance between the deleterious effects of most mutations and the cost of further increasing the fidelity of DNA replication (DRAKE 1991; DRAKE *et al.* 1998).

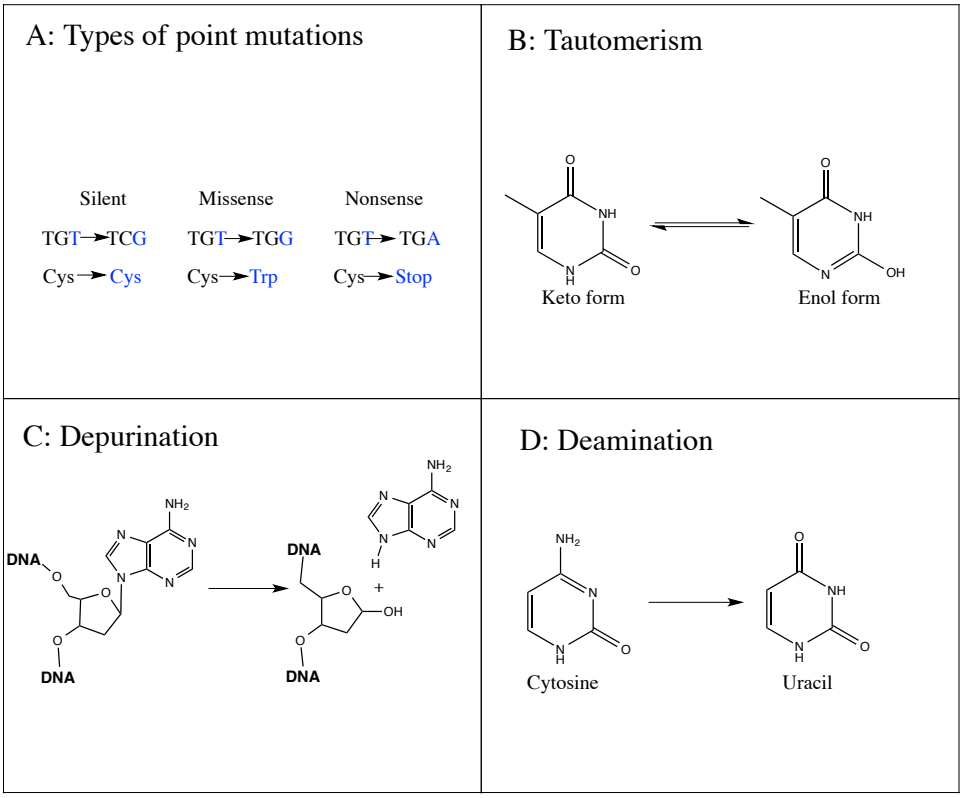

Figure 1: Intrinsic instabilities of DNA. A: Types of point mutations. Silent mutations change the base pair sequence, but the expressed protein remains the same. Missense mutations cause a change in the amino acid sequence, yielding a different protein. Nonssense mutations introduce stop codons, stopping synthesis of the protein entirely. B: Tautomers of thymine. DNA bases are originally in a keto form, which is required for correct Watson-Crick base pairing. C: Depurination. Purines, here represented by adenine, can be hydrolysed from the phosphatedeoxyribose backbone. D: Deamination of cytosine into uracil. Tautomeric, depurinated and missing bases can result in incorporation of wrong nucleotides during DNA replication and consequently can cause point mutations (adapted from SEEBERG *et al.* 1995).

1.2.1.1 Mutators

Since mutations are more likely to be deleterious than advantageous (DENAMUR and MATIC 2006), any variant of a clone with an increased mutation rate would *a priori* assumed to be less fit than its wildtype counterparts. Contrary to this intuitive expectation, bacterial strains with elevated mutation rates are not rare. In a study on 696 *E. coli* clinical isolates from Denmark, Sweden and Spain, 23% showed a slightly elevated mutation frequency ($>4*10^{-8}$) and 0,7% showed a strongly elevated mutation frequency ($>4*10^{-7}$) (BAQUERO *et al.* 2004). In another study on the *E. coli* strain O157:H7, five strains out of 120 screened had at least a 50-fold increase in mutation frequency (LECLERC *et al.* 1996). A phenotype conferring an increased mutation rate is termed *mutator*. The increase in mutation rate depends on the function lost to achieve a mutator phenotype, and can range from 10-fold to 10 000-fold (MILLER 1996). A mutation that leads to a mutator phenotype is not directly favoured by natural selection, but can be co-selected with another beneficial mutation if they are physically linked (DE VISSER 2002). If there is a large potential for beneficial mutations for a small population in an environment, the mutator subpopulation will prosper since they have a larger probability of acquiring a beneficial mutation before the wildtype population. Once a beneficial mutation has been acquired, natural selection will favour the mutator strain and mutator genes will spread within the population. In large populations, several clones in the mutator subpopulation will acquire the different beneficial mutations simultaneously. This leads to a phenomenon termed clonal interference, where the different mutants compete with each other within the population, increasing the fixation time for the most optimal mutation (GERRISH and LENSKI 1998). In sexual populations, loss of genes that cause a mutator phenotype when non-functional can be reacquired by homologous recombination, making the mutator phenotype a transient state (TENAILLON *et al.* 2000).

1.2.1.2. Deletions, duplication and amplification

A deletion is a loss of a segment of DNA that can be up to several thousand base pairs in size. Deletions can occur in several ways. Studies in *Escherichia coli* suggest that deletions frequently occur when a replication fork stalls due to a single strand or double strand break in DNA. The deletions of the DNA segments occur during the repair process through misalignment of complimentary single-stranded DNA segments at similar or identical DNA sequences (BIERNE *et al.* 1997). Deletions can also be mediated by DNA gyrase, which is an

enzyme that introduces negative supercoils in double-stranded DNA and is essential for DNA replication. Supercoiling is catalysed by binding to DNA at a consensus sequence and introducing a double strand break, flipping the ends by 360°, and religation of both strands. In rare cases, the DNA gyrase can join unrelated DNA ends after supercoiling, leading to a deletion of the fragment in between the joints. DNA segments can also be deleted by RecA-mediated recombination (IKEDA *et al.* 2004; MIURA-MASUDA and IKEDA 1990) and by other site-specific or –unspecific recombinases such as transposases through imprecise excision. Duplications and amplifications can arise by RecA-mediated (see below) exchanges between long repeats, or by RecA-independent exchanges between short or even absent repeats. DNA gyrase may also mediate gene amplification (reviewed in ANDERSSON and HUGHES 2009). Moreover, complex DNA alterations such as inversions and translocations can occur by the functions mentioned in this section.

1.2.2. Insertion sequences and transposons

Transposons are genetic elements that can induce mutations through site-specific recombination. A transposon consists of a gene coding for transposase, an enzyme that recognizes, cuts and inserts the transposon from one site to another, and two inverted repeats at the ends of the sequence. A transposon containing only inverted repeats and a transposase are termed insertion sequences (IS-elements). Transposons can also contain other genes, including genes encoding conjugation functions (conjugative transposons), different antibiotic resistance genes, or virulence genes (composite transposons). Transposons cause mutations by moving around the genome, and disrupting genes (reviewed in MAHILLON and CHANDLER 1998). Elena and colleagues showed in 1998 that 80% of 226 chloramphenicol-, tetracycline- or kanamycin-resistant *E. coli* mutants that had acquired the resistance phenotype through transposons had a significantly reduced fitness compared to wildtype, and the remaining 20% had a neutral or not measurable effect of fitness. These results suggest that mutation by insertion cause more harm on average than a set of random point mutations would (ELENA *et al.* 1998).

1.2.3. Recombination

Recombination is an event where two DNA strands are physically exchanged resulting in an inheritable alteration in DNA (REDFIELD 2001). Recombination occurs frequently between

similar or identical DNA molecules (homologous recombination) but also at considerably lower frequency between DNA molecules with little or no homology (IR).

1.2.3.1. Homologous recombination

Homologous recombination is an exchange of DNA strands at homologous stretches of DNA. The term homologous DNA is used for identical or highly similar sequence segments. RecA plays a key role in homologous recombination by binding to free, single-stranded DNA in the cytoplasm, then searching double-stranded DNA for homology. Once homology is found, RecA mediates invasion of the free strand into the double-strand and displacement of the corresponding strand. RecA deficiency leads to severe reduction in the frequency of homologous recombination (10^3- to 10^6-fold and sensitivity to DNA damaging agents such as mitomycin or UV (KOWALCZYKOWSKI *et al.* 1994). Exo- and probably endonucleases play a role by processing the displaced and the invading strands, so the invading strand can be ligated with the recipient strand. Several of the recombination enzymes overlap in function, creating different pathways for homologous recombination (Figure 2). The RecBCD enzyme contains both helicase and exonuclease function (reviewed in PERSKY and LOVETT 2008). During homologous recombination, RecBCD unwinds double stranded DNA and degrades one of the strands from the 5'-end, leaving the corresponding strand available for recombination by actively loading it with RecA protein. If *recBCD* is knocked out, other helicases such as RecQ and other exonucleases such as RecJ or RecE can replace the functions of RecBCD, still enabling homologous recombination as shown in *E. coli* (KOWALCZYKOWSKI *et al.* 1994) and *Acinetobacter baylyi* (KICKSTEIN *et al.* 2007). The DNA exchange through homologous recombination can be reciprocal, depending on the mechanism used to recombine the invading strand. Homologous recombination plays a major role in DNA repair, as RecA is expressed during the SOS response in *E. coli* (MARSH and WALKER 1987), which is triggered when severe genotoxic stress occurs. As a secondary function, homologous recombination can also lead to the introduction of new genes in a cells genome by recombining DNA obtained through horizontal gene transfer (see below) (reviewed in KOWALCZYKOWSKI *et al.* 1994; PERSKY and LOVETT 2008). Redundant enzymatic functions are not necessarily limited to recombinatory pathways: Single-strand-specific endonucleases not only process recombination intermediates, but also degrade the excised (mismatch-containing) single-strand in DNA mismatch repair (BURDETT *et al.* 2001; VISWANATHAN *et al.* 2001) and hydrolyse

free DNA ends in the cytoplasm (DUTRA *et al.* 2007).

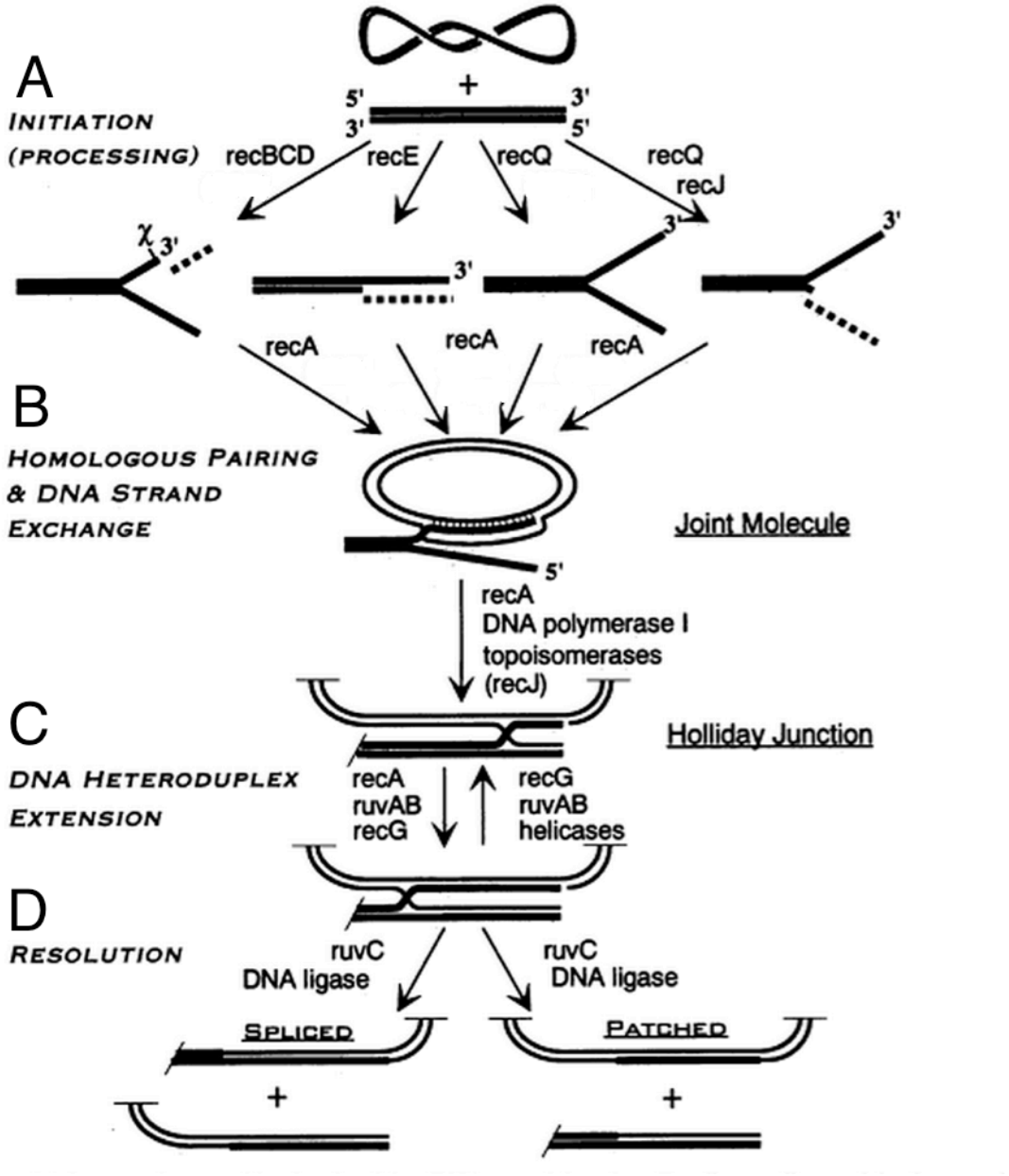

Figure 2: Mechanism and pathways of homologous recombination. A: double stranded DNA is initiated by either being unwound or degraded into single stranded DNA. B: single stranded DNA is loaded with RecA and a homology search is done. C: a heteroduplex structure is formed and a crossover occurs. D: the heteroduplex structure resolved and the two DNA strand are either spliced, or a reciprocal exchange occurred and they are both patched (adapted and modified from KOWALCZYKOWSKI *et al.* 1994).

1.2.3.2. Illegitimate recombination

Illegitimate recombination (IR) is a term used for recombination that is either not facilitated by RecA, or recombination between non-homologous DNA strands. Studies on IR have mainly been conducted employing four different experimental setups: excision and recombination of prophage Lambda DNA in *E. coli* (IKEDA *et al.* 2004), resolution of stalled replication forks in plasmid DNA containing two termination sites in *E. coli* (BIERNE *et al.* 1997), gene amplification by IR in *A. baylyi* (REAMS and NEIDLE 2003), or various constructs capturing homology-facilitated illegitimate recombination (HFIR) events in *A. baylyi* (DE VRIES and WACKERNAGEL 2002; HARMS *et al.* 2007; HULTER and WACKERNAGEL 2008a), *Pseudomonas stutzeri* (MEIER and WACKERNAGEL 2003), and *Streptococcus pneumoniae* (CLAVERYS *et al.* 1980; PRUDHOMME *et al.* 2002). IR events are differentiated by the presence or absence of micohomology between the donor and recipient strands. (IKEDA *et al.* 2004). The term microhomology describes small, random similarity between DNA strands, typically 3-8 base pairs (DE VRIES and WACKERNAGEL 2002). Bierne and co-workers showed that IR events without microhomology do not occur in an *E. coli* mutant with *topA10*, the gene transcribing topoisomerase 1 (DNA gyrase), knocked out. This result suggests that one mechanism of IR involves integration of foreign DNA by DNA gyrase (BIERNE *et al.* 1997). Microhomology-dependent IR is best described as part of HFIR, where a partly homologous and partly heterologous DNA strand is recombined (Figure 3)(DE VRIES and WACKERNAGEL 2002). DNA with a homologous segment with two heterologous sites at either side can be recombined by homology-facilitated double illegitimate recombination (HFDIR), although at lower frequencies than HFIR (HULTER and WACKERNAGEL 2008a). IR is considered a random and rare event, and does not have a clear function like the DNA repair functions of homologous recombination. It does enable acquisition of foreign DNA without participation of mobile genetic elements, which can increase diversity within a bacterial population.

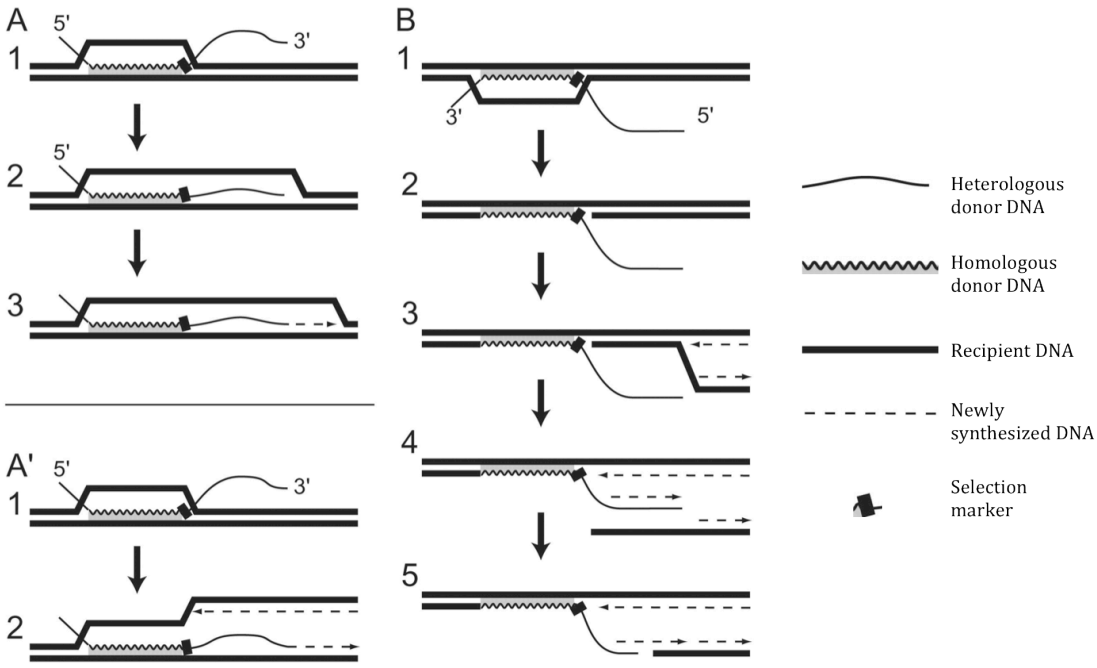

Figure 3: Mechanism of HFIR, where a DNA segment with one homologous part and one heterologous part is recombined. A: Integration of a heterologous 3' end by recombination at the leading strand. Microhomologies between the heterologous part and the recipient strand form a transient contact, which results in recombination once a replication fork runs through. A': Integration of a heterologous 3' end by recombination at the lagging strand. The heterologous 3' end is used as a primer for lagging strand synthesis. B: Integration of a heterologous 5' end. 1-2, the homologous anchor hybridizes at the recipient strand. 3-4, a replication fork approaches and the heterologous part of the donor DNA is converted to a double strand by acting as a template for a lagging strand. 5, a double strand break resulting in a 3'- single-stranded region extending from the Okazaki-fragment is formed. A double-strand break repair mechanism fuse the duplex donor and recipient DNA (adapted from DE VRIES and WACKERNAGEL 2002).

1.2.4. Horizontal gene transfer

Inter-genomic recombination occurs when bacteria acquire exogenous DNA. Acquisition of DNA for such inter-genomic recombination events is termed Horizontal Gene Transfer (HGT). The three most common mechanisms of HGT are transduction, conjugation and transformation. Other mechanisms such as nanotubes (DUBEY and BEN-YEHUDA 2011) and outer-membrane-vesicle mediated transfer (YARON *et al.* 2000) are described in the literature, but are beyond the scope of this thesis. All mechanisms of HGT enable bacteria to acquire genetic material from members of the same population or from otherwise distant relatives.

1.2.4.1. Conjugation and transduction

Conjugation is a cell-to-cell contact-dependent mechanism where a copy of a plasmid, a conjugative transposon or another mobile genetic element (MGE) is transferred from one bacterial cell to another (reviewed in CHEN *et al.* 2005; FROST *et al.* 2005). The MGE, typically plasmids, may encode genes for their own transfer, including a pilus that connects the donor with the recipient and all other proteins involved in the plasmid transfer. Conjugated MGEs can also contain beneficial properties, such as pathogenicity and nicer islands (GROHMANN *et al.* 2003). Conjugative transposons and plasmids play a major role in the spread of antibiotic resistance among bacteria (reviewed in SALYERS and AMABILE-CUEVAS 1997; VAN HOEK *et al.* 2011). Transduction is transfer of both chromosomal and extrachromosomal DNA horizontally by bacterial phages. Transducing phages are created as a result of errors in the lytic pathway of a phage, which leads to packing of bacterial host DNA inside a viral particle. If the transducing phage then infects a bacterial cell, the bacterial DNA will be injected into the recipient's cytoplasm, and can be recombined with the genome (reviewed in FROST *et al.* 2005). Successful transduction depends on the transducing bacteriophage target hosts, typically limiting DNA transfer to cells from the same species. The host range of transduction is narrow compared to conjugative genetic elements (MAJEWSKI 2001), although broad-host range bacteriophages exist (RUHFEL *et al.* 1984).

1.2.4.2. Natural transformation

Natural transformation is a process where DNA is actively taken up and integrated into the genome of so-called naturally competent bacteria. It was first observed in *Streptococcus pneumoniae* (GRIFFITH 1928). Transformation is at the core a two-step process: Active DNA uptake followed by genomic incorporation. For transformation to occur, the recipient bacterium needs to be in a physiological state termed competence. Competence can be constitutive as seen in *Neisseriae* and *Acinetobacter* and inducible as seen in *Bacillus subtilis* (LORENZ and WACKERNAGEL 1994). Competence can be a transient state, being activated when triggered by stress, during logarithmic growth, or by other specific conditions. The DNA uptake process is mediated by type IV pili. The translocation system spans the inner membrane and the cell wall in gram-positive bacteria, and the inner membrane, periplasm and outer membrane in gram-negative bacteria. (CHEN and DUBNAU 2004). Most bacteria take up DNA non-selectively, with at least two known exceptions, *Neisseria gonorrhoeae* and

Haemophilus influenzae, where the donor DNA is recognized by DNA uptake sequence receptors (DUS-R) on the cell surface, and only taken up if a matching sequence is found by the receptor (FRYE *et al.* 2013). In the uptake process into the cytoplasm, the transformed DNA is degraded to a single strand except in *H. influenzae* (GOODGAL 1982). Once in the cytoplasm, the DNA may be recombined with the chromosome, or, in the case of plasmids, restore its circular structure and subsequently be expressed (reviewed by LORENZ and WACKERNAGEL 1994).

1.3. *Acinetobacter baylyi* as a model organism

Acinetobacter baylyi is an aerobic, non-motile gram-negative soil dwelling coccobacillus of the *Moraxellaceae* family (CARR *et al.* 2003). The *Acinetobacter baylyi* strain ADP1 used in this study was originally a mutant of *Acinetobacter calcoaceticus* (now *baylyi*) strain BD4, a strain isolated from soil (JUNI and JANIK 1969). It is immediately transformable when transferred into a fresh rich medium, and is transformable at high frequencies (compared to many other organisms) by both homologous and heterologous DNA (PALMEN *et al.* 1993). The ADP1 genome has been sequenced (BARBE *et al.* 2004) (accession number NC_005966), and is circular, 3.6-mega base pairs long. The generation time of ADP1 is about 46 minutes in rich medium (HARMS and WACKERNAGEL 2008). ADP1 grows at 30-37^{o}C, and can utilize various different nutrients as carbon source, among them succinate. The available full sequence, combined with its easy handling, versatility and competence makes ADP1 suitable for the experiments done in this master thesis.

1.4. Short patch illegitimate recombination (SPIR)

Short Patch Illegitimate Recombination (SPIR) is a novel phenomenon that was discovered during an attempt to transform an *Acinetobacter baylyi* strain by ancient DNA (Overballe-Petersen *et al.,* manuscript submitted). This strain contained a 213-bp insert of random sense codons (with the exception of two consecutive stop codons) in its *hisC* gene. The stop codons prevent expression of that gene, resulting in inability to synthesize histidine.

DNA sequencing of false-positive, histidine-proficient isolates revealed recombinants with the two consecutive stop codons replaced by sense codons accompanied by further DNA sequence changes that are problematic to explain by point mutations. We hypothesize that

these nucleotide exchanges were created as the result of an IR event with heterologous DNA.

1.5. Aim

To characterize the phenomenon of short patch illegitimate recombination (SPIR) in *Acinetobacter baylyi.*

Hypotheses to test

Short Patch Illegitimate Recombination (SPIR) is a class of mutations where heterologous DNA is recombined independently of RecA, but not of RecJ and ExoX. Natural transformation can provide diverse DNA for recombination, but is not mandatory for SPIR to occur.

Specific objectives

To characterize different types of SPIR mutants in KOM218, an *Acinetobacter baylyi* strain containing two consecutive stop codons in *hisC.*

To investigate the influence of RecJ, RecA and ComA on SPIR events.

2. Materials and methods

2.1. Materials

2.1.1. Bacterial strains

All strains used in this thesis originate from the *Acinetobacter baylyi* ADP1 strain (JUNI and JANIK 1969) (see introduction for more details) and are listed in Table 1. Strain KOM218 carries the SPIR detection construct *hisC*::'ND5i', is auxotrophic for histidine and does not grow on M9 minimal medium. The genes *recJ* (encoding the 5'-single-stranded DNA-specific exonuclease RecJ) and *exoX,* (encoding the 3'-single-stranded DNA-specific exonuclease ExoX) are deleted in KOM218. The non-transformable strain AL1 is derived from KOM218 and has a similar genotype, except *comA* (coding for the DNA uptake pore ComA) has been substituted by the selective/counterselective marker pair *nptII sacB* (conferring resistance towards kanamycin and sucrose susceptibility). Strain JV28 is prototrophic for histidine but auxotrophic for tryptophan *(trpE27)*. Strain AL2 is isogenic to JV28, except that the *trpE*$^{+}$ allele has been restored, making AL2 prototrophic for tryptophan and growth-proficient on M9 minimal medium. Strain AL3 is isogenic to AL2, except that the *nptII sacB* genes have insertionally inactivated the *hisC* gene. Strain AL4 is derived from AL3, where the *nptII sacB* segment in *hisC* is substituted by a 213 base pair segment of random sense codons except for two consecutive stop codons approximately in the centre of this segment ('ND5i'). Strain AL5 is derived from AL4, and in this strain the chromosomal *recJ* gene has been replaced by the nptII sacB marker pair. In strain AL6, the marker pair has been deleted, resulting in a marker-free Δ*recJ* allele in this strain. In addition, all strains carry the *alkM*::(*nptII'* tg4) insertional inactivation that blocks utilization of long-chain alkanes as carbon source, and the *rpoB1* mutation that confers resistance to rifampicin. In a single control experiment, DNA from the ADP1 wildtype strain JV28 was used as donor DNA for transformation.

The *Escherichia coli* strain EC100 (Epicentre, WI, USA) was used as host for the plasmids used to construct the various AL-strains.

Table 1: List of strains used in this thesis.

Name	Genotype	Reference
KOM218	ADP1 *hisC*::'ND5i' Δ*recJ* Δ*exoX rpoB1* (Rif^r) *alkM::*(*nptII'* tg4)	Overballe-Petersen *et al.,* manuscript submitted
JV28	ADP1 *trpE27 rpoB1*(Rif^r) *alkM::(nptII'tg4)*	de Vries *et al.,* 2003
AL1	ADP1 *hisC*::'ND5i' Δ*recJ* Δ*exoX* Δ*comA rpoB1* (Rif^r) *alkM*::(*nptII'* tg4)	This study
AL2	ADP1 *trpE$^+$ rpoB1*(Rif^r) *alkM*::(*nptII'* tg4)	This study
AL3	ADP1 *hisC::*(*nptII sacB*) (Kan^r Suc^s) *rpoB1* (Rif^r) *alkM::*(*nptII'* tg4)	This study
AL4	ADP1 *hisC*::'ND5i' *rpoB1* (Rif^r) *alkM::*(*nptII'* tg4)	This study
AL5	ADP1 *hisC*::'ND5i' Δ*recJ*::(*nptII sacB*) *rpoB1* (Rif^r) *alkM::*(*nptII'* tg4)	This study
AL6	ADP1 *hisC*::'ND5i' Δ*recJ rpoB1* (Rif^r) *alkM::*(*nptII'* tg4)	This study

2.1.1.1. Freeze stocks

After at least two steps of purification on appropriate solid media were single colonies from the various *A. baylyi* ADP1 mutants harvested with a sterile 1 µl loop, suspended in 1 ml LB+20% glycerol in a Falcon freeze tube (VWR international, USA), and stored at -75^{o}C.

2.1.2. Media

Liquid and solid media was autoclaved at 121^{o}C for 20 minutes.

2.1.2.1. Complex medium

The complex medium of choice for this thesis was *Luria-Bertani (LB)* broth, which consists of 10 g trypton (BD Difco, USA), 5 g yeast extract (Fluka Sigma-Aldrich, Germany), 10 g NaCl (Fluka Sigma-Aldrich, Germany) per 1 litre of demineralized water (H_2O). In liquid media, the pH was adjusted to 7.5 by using NaOH 10M before autoclavation. In solid media, 15 g of agar (Fluka Sigma-Aldrich, Germany) were added before autoclavation, with no pH adjustment. Liquid media was stored in the bottle it was autoclaved in, and used for making

liquid cultures. Solid media was cooled down to approximately 60°C after autoclavation and poured into aseptic petri dishes. Solid media with sucrose was created in the same way as normal LB plates, but with 50 g sucrose added before autoclavation in addition to the other ingredients. Solid media with kanamycin was made by adding 400 µl/l of a kanamycin stock solution (25g/l) to the media after autoclavation.

2.1.2.2. Minimal medium

The defined medium of choice was M9 minimal medium. It does not contain histidine, making it suitable for selection of *hisC* mutants. M9 minimal medium was made by mixing 15 g of agar and 800 ml of demineralized water, autoclaving it, and then adding 0,5 ml of 200mM $CaCl_2$ (Sigma-Aldrich, Germany), 2 ml of 1M $MgSO_4$ (Merck, Germany), and 200 ml of M9 salts. Unless noted otherwise, 10 ml sodium-Succinate (Sigma-Aldrich, Germany), pH 7.5, were added as carbon source. The composition of M9 salts were: 85,49 g/l $Na_2HPO_4 \times 12H_2O$ (Sigma-Aldrich, Germany), 15 g/l KH_2PO_4 (Sigma-Aldrich, Germany), 2,5 g/l NaCl (Sigma-Aldrich, Germany), 5 g/l NH_4Cl (Sigma-Aldrich, Germany). All aqueous solutions were autoclaved at 121°C for 20 minutes before mixing with the agar solution. In one control experiment, M9 plates were supplemented with histidine as follows: Before plate pouring, 2 ml of a sterile-filtered aqueous 1% solution (w/v) of histidine (obtained from K. Harms) was added to the M9 medium.

2.1.3. Solutions

2.1.3.1. Antibiotics

Stock solutions for kanamycin (25g/l dH_2O), chloramphenicol (25g/l EtOH) and ampicillin (200g/l dH_2O) were provided by Klaus Harms. Kanamycin is an aminoglycoside that inhibits the 30S subunit in the ribosome, interfering with translation of RNA (KOTRA *et al.* 2000). Chloramphenicol inhibits peptidyltransferase in the 50S subunit, preventing protein synthesis (SCHWARZ *et al.* 2004). Ampicillin is a beta-lactam antibiotic that inhibits synthesis of the peptidoglycan wall in gram-positives and –negatives (KOTRA and MOBASHERY 1998).

2.1.3.2. Buffers and further solutions

Phosphate buffered saline (PBS) was used for re-suspension and washing of cells before spreading on M9-plates. PBS consisted of: 8 g/l NaCl (Fluka Sigma-Aldrich, Germany), 0,2 g/l KCl (Merck, Germany), 3,63 g/l $Na_2HPO_4 \times 12\ H_2O$ (Sigma-Aldrich, Germany), and 0,24 g/l KH_2PO_4 (Sigma-Aldrich, Germany). pH was adjusted to 7.4. The buffer was autoclaved at 121°C for 20 minutes.

Tris-buffer 1M, pH 8.0 was made by adding 12,1 g of Trizma base (Sigma-Aldrich, Germany) to 100 ml of demineralized water, pH was adjusted to 8.0 by concentrated HCl. The buffer was autoclaved at 121°C for 20 minutes.

1×TAE buffer was used for gel electrophoresis. A 50× stock solution was made by dissolving 482 g Trizma base (Sigma-Aldrich, Germany) in 1600 ml demineralized water, followed by the addition of 114,2ml Glacial Acetic acid and 100ml 0.5M EDTA (Merck, Germany). Demineralized water was added up to 2L. This stock solution was then diluted to 1× in aliquots.

ExoSap-I was used for purification of PCR products. It was made using the following recipe: 1 µl Exonuclease I, 2 µl SAP (Shrimp Antarctic (Alkaline) Phosphatase), 2 µl SAP-buffer and 15 µl distilled water (dH_2O). SAP-buffer was composed of 100 µl 1M $MgCl_2$, 200µl 1M Tris-HCl pH 8.0, and 700 µl demineralized and autoclaved H_2O.

Buffers used for DNA isolation and purification steps (QIAGEN, Germany):

Buffer B1 (Lysis buffer): 50mM Tris-Cl, pH 8.0; 50mM EDTA pH 8.0; 0,5% Tween-20; 0,5% Triton X-100.

Buffer B2 (Lysis buffer): 3M Guanidine HCl; 20% Tween-20.

Buffer QBT (Equilibration buffer): 750mM NaCl; 50mM MOPS pH 7.0; 15% isopropanol; 0,15% Triton X-100.

Buffer QC (Wash buffer): 1,0M NaCl; 50mM MOPS pH 7.0; 15% isopropanol.

Buffer QF (Elution buffer): 0,25M NaCl; 50mM Tris·Cl pH 8.5; 15% isopropanol.

Buffer P1 (Resuspension buffer): 50mM Tris·Cl, pH 8.0; 10mM EDTA; 100µg/ml RNase A.

Buffer P2 (Lysis buffer): 200mM NaOH; 1% SDS

Buffer P3 (Neutralization buffer): 3M Potassium acetate (CH_3CO_2K) pH 5.5.

Buffer TE (DNA resuspension and storage buffer): 10 mM Tris·Cl, pH 8.0; 1mM EDTA.

2.1.4. Primers

Table 2 contains a list of primers used for PCR amplification and sequencing in this thesis.

Table 2: Primers used in this thesis. Lower case letters indicate 5'-heterologous tail.

Name	Sequence 5' - 3'	Source
comA-up-f	gTAAAATTTTTATGGTGCAGG	Overballe-Petersen *et al.,* manuscript submitted
comA-down-r3	aactTGAATCGCTCCCTTATCAGTTG	Overballe-Petersen *et al.,* manuscript submitted
hisC-up-f	AATCTGGATGCACATGCACG	Overballe-Petersen *et al.,* manuscript submitted
hisC-ins-r	CAATTACGACTACACGATCG	Overballe-Petersen *et al.,* manuscript submitted
recJ-up-f	gtaAGCCTTCTGGACATATTTTGACC	Kickstein *et al.,* 2007
recJ-down-r	aacttgACTTACATTATTACAGTTCAGTTACG	Kickstein *et al.,* 2007

2.2. General methods

2.2.1. Plasmid DNA isolation

Plasmid DNA isolation from *E. coli* EC100 strains was done according to the instructions in the QIAGEN plasmid DNA isolation handbook (QIAGEN, Germany). Briefly, a single colony from each of the plasmid-containing EC100 strains was inoculated in 10 ml LB broth. In the cultures containing pKHNH2, pKHhisC20 and pEK2, 25 mg/l kanamycin was added. With pKHhisC25 and pEK3, 10 mg/l chloramphenicol was added. After 16 hours of incubation at 37^{o}C, the cells were pelleted by centrifugation at 6000×g at 4^{o}C for 15 minutes. The pellet was resuspended in 1 ml of buffer P1 with RNase A (100µg/ml) added and vortexed. Afterwards 1 ml buffer P2 was added, and the tube inverted a few times, causing the cells to lyse. The tubes were incubated at room temperature for 5 minutes. Then 1 ml of pre-cooled buffer P3 was added, which caused a white precipitate of cell debris and genomic DNA to form. The debris was spun down at 4000 RPM, 4^{o}C for 30 minutes. The supernatant was then harvested and loaded onto a 20/G QIAGEN tip that had been previously treated with 1 ml of buffer QBT. The supernatant was collected and run through the tip twice. Subsequently 2 ml of buffer QC was run through the tip to wash the DNA. Then 1 ml of buffer QF was run through the tip and gathered in an eppendorf tube. 0.7 ml of isopropanol was added to the eppendorf tube, and then the mix was spun down in a centrifuge at 13,000 RPM at 4^{o}C for 60 minutes. The supernatant was discarded, and the plasmid pellet was rinsed with 1 ml of ethanol. The eppendorf tubes were then dried at 37^{o}C until all of the ethanol had evaporated, and finally the plasmid DNA pellet was suspended in 20 µl of TE buffer.

2.2.2. Genomic DNA isolation

Genomic DNA isolation was done as described in the QIAGEN Genomic DNA Handbook (QIAGEN, Germany). A liquid culture of *Bacillus subtilis* was prepared by inoculating a single colony in 10ml of LB media. The culture was then grown for 16 hours at 37^{o}C, and spun down in a pre-cooled 4^{o}C centrifuge for 10 minutes at 4000 RPM. The supernatant was decanted, and the pellet suspended in 3,5 ml of buffer B1. Then 7µl of RNase A (100 µg/ml), 80 µl of lysozyme (100 mg/ml) and 150 µl of proteinase K (20 mg/ml) was added to the assay. The tube was then incubated in a 37^{o}C water bath for 30 minutes followed by the

addition of 1,2 ml of buffer B2 . The tube was further incubated in a 50°C water bath for 30 minutes. 3,5 ml of isopropanol was added and the tube was inverted repeatedly. The precipitated genomic DNA was harvested from the tube with a sterile 1 µl loop (dipped in 1 ml ethanol). The tube was dried at 37°C for 2 hours. The dried DNA in the eppendorf tube was suspended in 100 µl of buffer TE and stored at -20°C for later use.

2.2.3. Transformation assays

Acinetobacter baylyi is highly transformable in fresh liquid LB medium when it is inoculated from a stationary phase culture (PALMEN *et al.* 1993). The transformation assays used to construct the strains in this study was done by first growing a 10 ml liquid culture in LB with *Acinetobacter baylyi* inoculated from solid medium for 16 hours. This culture was spun down with a centrifuge at 4000 RPM, 4°C for 8 minutes. The supernatant was decanted, and the pellet was suspended in 6 ml of fresh LB medium. 1 ml of the suspended culture was transferred to a glass tube, where 10 µg of linearized plasmid DNA was added. The glass tube was then incubated at 30°C in a shaker for 90 minutes at 150 rpm. After incubation, 100 µl of the culture was streaked out on solid media with an appropriate selection marker, and grown for 24 hours at 30°C. Single colonies were picked from this plate and purified on a new plate with the selection marker for one more day. This purification process was repeated to ensure that a single clone had been isolated.

2.2.4.Strain constructions

In this section the construction of the bacterial strains listed in Table 1 is described.

2.2.4.1. Strain AL1

Strain AL1 was constructed by natural transformation using the plasmid DNA construct pKHNH2 (Table 3), which contains a *nptII sacB* marker pair, embedded in the native flanking chromosomal regions of the *comA* gene and thus substituting *comA*. pKHNH2 was purified (section 2.6) and treated with the restriction enzyme *Xho*I (New England Biolabs, USA) prior to transformation. Strain KOM218 was transformed by linearized pKHNH2 (Table 3), and transformants that have recombined with the DNA and replaced *comA*$^+$ with the *nptII sacB* genes were selected on LB with 10 mg/l kanamycin. Colonies were picked with a sterile loop

and restreaked on kanamycin-containing LB medium. From that plate, from all streaks a single colony was picked, restreaked on medium with kanamycin and as control on medium with 50g/l sucrose. Isolates that formed colonies on kanamycin but not on sucrose-containing media were verified to contain the desired D*comA*::(*nptII sacB*) allele by PCR (see section 2.9). The PCR reaction was done with the primers *comA*-up-f and comA-down-r3 (Table 2), yielding a 3411 bp PCR product. One isolate was termed AL1 and used for experiments.

Table 3: List of plasmids and their relevant properties used in this thesis.

Name	Relevant properties	Reference
pKHNH2	pGT41 containing Δ*comA*::(nptII sacB)	Overballe-Petersen et al., manuscript submitted
pUC19-trpE	pUC19 containing *trpE*$^+$	K. Harms, unpublished
pKHhisC20	pACYC184 containing upstream segment and 5'-terminus of *hisC*::(*nptII sacB*)	Overballe-Petersen et al., manuscript submitted
pKHhisC25	pACYC184 containing upstream segment and 5'-terminus of *hisC*::'ND5i'	Overballe-Petersen et al., manuscript submitted
pEK2	pGT41 containing Δ*recJ*::(*nptII sacB*)	Kickstein et al., 2007
pEK3	pGT41 containing border sequences of Δ*recJ*	Kickstein et al., 2007

2.2.4.2. Strains AL2, AL3 and AL4

Strain AL2 was also constructed by natural transformation. Here the plasmid pUC19-trpE5 was used, which contains the *trpE*$^+$ allele to restore the wildtype allele in the strain JV28, which carries the *A. baylyi trpE27* allele. The plasmid was linearized with restriction enzyme *Sca*I (Fermentas, Germany) before transformation. After transformation, the cells were centrifuged for 6 min at 5000×g and 4°C before plating on minimal medium. Colonies were picked and isolated as described above. Since this step was performed to restore the tryptophan prototrophy, no verifications by PCR or sequencing were made.

Strain AL3 was created by transformation with pKHhisC20. This plasmid contained a segment overlapping with the *A. baylyi hisC* allele with the *hisC* insertionally inactivated by

the *nptII sacB* marker pair genes. pKHhisC20 was treated with the restriction enzyme *Xmn*I (New England Biolabs, USA) before transformation. Kanamycin-resistant (and sucrose-sensitive) transformants were confirmed by PCR, verifying the presence using the primers hisC-up-f and hisC-ins-r (Table 2) to yield a 3432 bp PCR product.

Strain AL4 was created by transformation of the AL3 by pKHhisC25. This plasmid carries the *hisC*::'ND5i' allele (Table 3). *Xmn*I was used to linearize the pKHhisC25 before transformation. Transformants were scored on medium containing sucrose and screened for absence of kanamycin resistance. (PCR). One clone was termed AL4 and used for experiments.

2.2.4.3. Strains AL5 and AL6

In order to delete the *recJ* gene from AL4, this strain was subsequently transformed by plasmid pEK2 and plasmid pEK3. pEK2 contains a D*recJ*::(*nptII sacB*) allele embedded in its natural flanking regions, and pEK3 contains a corresponding D*recJ* allele. Selection for resistance to kanamycin (construction of AL5) and to sucrose (AL6) was performed according to the construction of AL3 and AL4 descriptions above. D*recJ*::(*nptII sacB*) clones were verified to contain the desired *recJ* substitution allele by PCR using the primers recJ-up-f and recJ-down-r (Table 2), which gave the expected 4.7 kbp PCR product (wildtype: 3.7 kbp). The PCR product of the D*recJ* isolates was 2.0 kbp as expected. One *recJ* deletion clone was termed AL6 and used for experiments.

2.2.5. PCR

PCR (Polymerase Chain Reaction) is a process where a specific DNA sequence is copied to ample numbers by specific *in vitro* DNA replication. PCR is performed using a heat-resistant DNA polymerase, together with the four deoxyribonucleoside triphosphates (dATP, dCTP, dTTP and dGTTP) and a pair of primers that specifically binds to the sequence that is amplified (MULLIS 1990). Three steps are critical for PCR: Denaturation of the DNA molecules, annealing of the primers to the DNA strands, and an elongation step where the DNA polymerase binds to the annealed primers and synthesizes the novel strand. The three steps operate at different temperatures (94°C for denaturation, 5°C below the primers melting point for annealing, and 72°C for elongation). Once one full cycle is finished, the newly synthesized strand can serve as template in the next cycle, making it an exponential

process. After PCR is completed, the samples are stored at 4°C. This is due to the endonuclease activity of the DNA polymerase. The PCR thermocycler program used in this study is described in Table 4.

Table 4: General PCR thermocycler program used in this study.

Step	Temperature	Time	Notes
1	94°C	5 minutes	
2	92°C	30 seconds	
3	58°C	30 seconds	
4	72°C	Varies	30 seconds per 500bp in the PCR product
5	Repeat step 2-4 30 times		
6	72°C	5 minutes	
7	4°C	For ever	

2.2.5.1. DreamTaq master mix

In this study, DreamTaq PCR master mix 2x (Fermentas, Germany) was used. It contains: DreamTaq DNA Polymerase, 2X DreamTaq buffer, dNTPs, and 4 mM $MgCl_2$. The complete assay consisted of 10 µl DreamTaq master mix, 1 µl of an approximately 10^8 cells/ml suspension, 0,2 µl of a matching set of primers, and 8,6 µl of distilled water (dH_2O).

2.2.5.2. Purification of PCR products

All PCR products were purified of unwanted primers with the ExoSap-I (see 2.1.3.2.) before they were sequenced. The ExoSap-I contains an exonuclease that degrades the primers added to the PCR assay. 2 µl of ExoSap-I and 5 µl of PCR product are mixed together, and then warmed up to 37°C for 30 minutes. Then the mix is heated to 80°C for 15 minutes to inactivate the exonuclease.

2.2.6. DNA sequencing

A sequencing assay contains a primer, a DNA polymerase, a DNA template, the standard deoxyribonucleotides (dNTPs: dATP, dGTP, dTTP and dCTP) and dideoxyribonucleotides (ddNTPs), which are unable to form phosphodiester bonds at the 3'-end. ddNTPs are fluorescently labelled, and each emit different wavelengths. When the DNA template is

sequenced various fragments of different lengths are formed depending on where in the DNA template the ddNTP was incorporated by the DNA polymerase. These fragments can be separated by size in an AB1 sequencer. By detecting the wavelength the differently sized fragments emit, the specific sequence can be identified (SANGER *et al.* 1977). The sequencing assay used in this thesis was 2 µl BigDye v3.1 (Life Technologies, USA), 3 µl BigDye buffer (Life Technologies, USA), 0,3 µl primer, 13,7 µl distilled water (dH_2O) and 1 µl of a purified PCR product. The sequencing reaction was run as shown in Table 5 and analysed on a sequencing machine (Genetic Analyzer 3130XL, Life Technologies) in the sequencing facility of the University of Tromsø (Faculty of Health). Sequencing results were obtained as *.ab1 files and analysed by BLAST (bl2seq, http://blast.ncbi.nlm.nih.gov/BlastAlign.cgi)

Table 5: General sequencing program used in this thesis.

Step	Temperature	Time
1	96°C	3 minutes
2	96°C	15 seconds
3	50°C	15 seconds
4	60°C	4 minutes
5	Repeat step 2-4 30 times	
6	4°C	For ever

2.2.7. Gel electrophoresis

Gel electrophoresis allows DNA molecules to be separated based on size. An agarose gel is cast, and the DNA samples are applied on top of it. Then an electrical current is applied to the gel. DNA is negatively charged, and will thus migrate towards the anode. The length of this migration depends on the size and conformation of the DNA molecule. By adding ethidium bromide to the gel, the bands visualize by emitting fluorescence light when irradiated by UV. The gels ran in this thesis consisted of 0,56 g agarose (SeaKem, USA), 80 ml 1×TAE buffer and 15 µg ethidium bromide (Sigma-Aldrich, Germany). DNA samples consisted of 2 µl 6× loading buffer, 9 µl of distilled water (dH_2O) and 1 µl of an isolated DNA preparation. For reference a molecular marker was used (SmartLadder, Eurogentec). All gels were run for 45 minutes at 95 volts. The gels were visualized using a Gel Doc 2000 transilluminator (BioRad, Norway) and the software Quantity One (BioRad, Norway).

2.3. Experimental methods

2.3.1. Determination of mutation frequency

Mutation frequency is here defined as the amount of mutant cells divided by the total amount of cells in a culture. First, a 20 ml overnight culture was inoculated and grown for 20 hours. Then it was spun in a centrifuge down at 4000 RPM, 4°C for 12 minutes. The supernatant was decanted, and the pellet suspended in 2 ml of PBS. The cell suspension was again spun down in a centrifuge at 4000 RPM, 4°C for 12 minutes. The supernatant was decanted, and the pellet was resuspended in 2 ml of fresh PBS. 50 µl of the fresh suspension was diluted to 10^{-6} the original concentration, and then 20 µl of the final dilution was plated on an LB plate. The remaining undiluted suspension was plated on 8 M9 plates. The colonies on the LB plate were counted after 24 hours of growth at 30°C. The colonies on the M9 plates were counted after 48 hours of growth at 30°C. These were purified further, and used for sequencing as well.

3. Results

3.1. SPIR-capturing construct

The construct used to detect Short Patch Illegitimate Recombination (SPIR) events was the *hisC*::'ND5i' allele of *Acinetobacter baylyi* KOM218, resulting auxotrophy for histidine (His). In strains with this construct, the *hisC* allele carries a 213 base pair-insert *in frame* named ND5i containing a set of two consecutive stop codons. These stop codons prevent expression of a functional HisC enzyme. Prototrophy for histidine can be restored typically either by a deletion, or by an SPIR event as evident from the results presented in this thesis. Natural transformation of a functional *hisC*$^+$ allele could possibly yield His$^+$ isolates at higher frequencies than both SPIR and deletions provided that the donor DNA is available. In order to avoid this kind of DNA contamination, all equipment is either was single use only, or treated with bleach. A personal stock of chemicals was used for these experiments to further avoid contamination of *hisC*$^+$ wildtype DNA.

3.1.1. His$^+$ frequencies of KOM218

To determine the His$^+$ frequency of KOM218 individual cultures were inoculated from single colonies and grown for 20 hours, then washed and plated on M9 medium with succinate. The cell titers of the cultures were determined by plating an appropriate dilution on LB medium. The LB plates were grown at 30^{o}C for 24 hours, and the M9 plates were grown for 48 hours. After growth, the colonies on the plates were counted. Typically, a 20 ml culture gave zero to four colonies. The mutation frequency was calculated by dividing the titer of His$^+$ colonies by the CFU titer. When no His$^+$ colonies were obtained, the detection limit was calculated. A total of 22 mutation frequency experiments were done with the KOM218 strain, and the median His$^+$ frequency was found to be 4.0×10^{-11} (Table 6).

Table 6: Overview of frequency data and SPIR %

Strain	Genotype	Median His$^+$ frequency	SPIR %
KOM218	Δ*recJ* Δ*exoX*	4.0×10^{-11}	32
AL1	Δ*comA* Δ*recJ* Δ*exoX*	7.3×10^{-11}	30
AL4	wildtype	$<5.0\times10^{-11}$	4
AL6	Δ*recJ*	1.2×10^{-10}	4

3.1.2. Characterization of His$^+$ mutants by DNA sequencing

His$^+$ colonies were picked and restreaked on M9-succinate medium, and the recombinant region of the *hisC*::'ND5i' allele was amplified by PCR, purified, and sequenced. The sequences were aligned with the sequence from the *hisC*::'ND5i' allele (FASTA file provided by K. Harms, Appendix 1) and the ADP1 genome sequence (accession number NC_005966) using the BLAST tool bl2seq. A total of 56 individual His$^+$ isolates of KOM218 were sequenced. When the sequences from two or more isolates from the same experiment were identical, they were considered as offspring cells from a single His$^+$ mutant and counted as single event (Table 6, right column). Two classes of His$^+$ mutant formations were identified; deletions and SPIR.

3.1.2.1. His$^+$ deletion mutants

In the KOM218 strain, 37 out of 56 His$^+$ isolates were formed by deletions ranging from 9 to 180 base pairs in the 'ND5i' segment that spanned the double stop codons and restored a correct reading frame. By aligning the His$^+$ isolate sequence with the 'ND5i' insert sequence, two types of deletion mutants were identified. In type 1, the deletions formed at microhomologies (short stretches of 3 to 18 identical or near-identical nucleotides at both ends of the recipient segment). In type 2, no microhomologies (none or one matching nucleotide at both ends of the recipient segment) were identified (see Table 7, Figure 4). These results suggest that the deletions occurred by different mechanisms. This is in agreement with findings in other organisms (BIERNE *et al.* 1997; IKEDA *et al.*

2004).

Table 7: Overview of deletion mutants found in this thesis.

Strain	No microhomology	3 or more bases matching	Total deletions
KOM218	19	18	37
AL1	7	9	16
AL4	15	9	24
AL6	12	10	22

3.1.2.2. His$^+$ SPIR mutants

18 out of 56 (32%, see Table 1) KOM218 His$^+$ isolates were formed by SPIR, with 7 different prototypes recurring in independent experiments (Figure 5-7). In all cases, an unrelated segment from the *A. baylyi* genome could be identified that substituted the two consecutive stop codons *in frame*. Upon closer analysis, three classes of SPIR mutants could be identified (Figure 5-7). The first class carried a single continuous patch covering the consecutive stop codons with presumptive crossovers upstream and downstream, resulting in several exchanged nucleotides but no deletions. The second class contained small deletions (3-9 nucleotides) together with several nucleotide exchanges that typically are aligned poorly by BLAST, with the exception of short, near-identical sequences at the borders (putative recombinant joints). The third class appeared as a larger deletion, bordered by near-identical segments at each side, with few exchanged nucleotides between them. One particular class 1 SPIR prototype (26, see Figure 5) recurred a total of 6 times independently in KOM218. The near-identical segments resemble microhomologies found previously in illegitimate recombinant joints in *A. baylyi* (DE VRIES and WACKERNAGEL 2002; HARMS *et al.* 2007; HULTER and WACKERNAGEL 2008a; REAMS and NEIDLE 2004) and other organisms. To assess the strength of patch microhomologies in the SPIR mutants, the minimum free energy of hybridization (ΔG^0_{min}) was calculated using the nearest neighbour method and base pair parameters given by Wetmur (1996).

The results are shown in Table 8.

Table 8: Overview of the minimum free energy to the patches found in this project. Full sequence alignments are shown in Figure 5-7.

Class	Prototype	ΔG^0_{min} (first or only patch)	ΔG^0_{min} (second patch)	Patch sequence	Strains (frequency)
1.	6.	-17.62		**CACCAGCCATAGCAGGCCCT**	KOM218 (4)
1.	26.	-22.86		**TTCCACCTGCAGCATAAGGCCCTAC**	KOM218 (6), AL1 (5)
1.	54.	-15.19		**CACCAGCCATAGCAGGCCCT**	KOM218 (2)
1.	60.	-10.01		**GCTATTTAAGGC**	AL1 (1)
2.	10.	-7.89	-8.24	**TCAGCTA**-TTGCGTGGTGGTGGTGCATT-**CTTCATTC**	KOM218 (1)
2.	17.	-7.85	-14.50	**ATCAGCT**-TGAAGCCGT-**GTATCAGCACT**	KOM218 (1)
2.	19.	-7.88	-12.91	**CCATCAG**-TTATCGTCGTCACT-**GGATCAGCACT**	KOM218 (1), AL1 (1)
3.	4.	-8.66	-16.77	**TAGAGCCTT-ATGTACCTGGTGAG**	KOM218 (2)
3.	100.	-11.87	-9.46	**TGACTTCCA-ATAGCTGTTGTAT**	AL4 (1)
3.	103.	-10.60	-7.40	**TTTGTACTTCA-TTCCAGAAC**	AL6 (1)

Type 1: Deletion mutant with microhomology.

```
A   ACTCCCACTACTAGGTCTTCTCTTAGCCTCAGCAGGAAAATCAGCCCAATTTGGACTTCATCCGTGACTTCCATCAGCTAGTGAAGGCCCTACCCCAGTATCAGCACTCCTTCATTCCAGTACAATAGTTATAGCTGGAGTATTTACCCTCATCCGCTTTTATCCATTAATAGAAAACAACCTCACTATTCAAACTTCAACACTTCTGGTTCTGGC
    ||||||||| || | |||                                                                                                                                                                                                   |||
R   ACTCCCACTTCTGGTTCT---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------GGC
```

Type 2: Deletion mutant with no microhomology.

```
A   CAGGAAAATCAGCCCAATTTGGACTTCATCCGTGACTTCCATCAGCTAGTGAAGGCCCTACCCCAGTATCAGCACTCCTTCATTCCAGTACAATAGTTATAGCTGGAGTATTTACCCTCATCCGCTTTTATCCATTAATAGAAAACAACCTCACTATTCAAACTTCAACACTTCTGGTTC
    ||||||||||||||||||||||||||||                                                   |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
R   CAGGAAAATCAGCCCAATTTGGACTTCA---------------------------------------------------TCATTCCAGTACAATAGTTATAGCTGGAGTATTTACCCTCATCCGCTTTTATCCATTAATAGAAAACAACCTCACTATTCAAACTTCAACACTTCTGGTTC
```

Figure 4: Deletion mutant examples. The type 1 deletion mutant has recognizable microhomology (including 3 unmatched base pairs). 13 base pairs (marked in blue) at the end of the deletion match the segment of 13 base pairs before the deletion. No microhomology was identified in the type 2 deletion mutant. A: ancestral *hisC*:: 'ND5i' sequence. R: sequence of His$^+$ isolate. The two consecutive stop codons are marked in red.

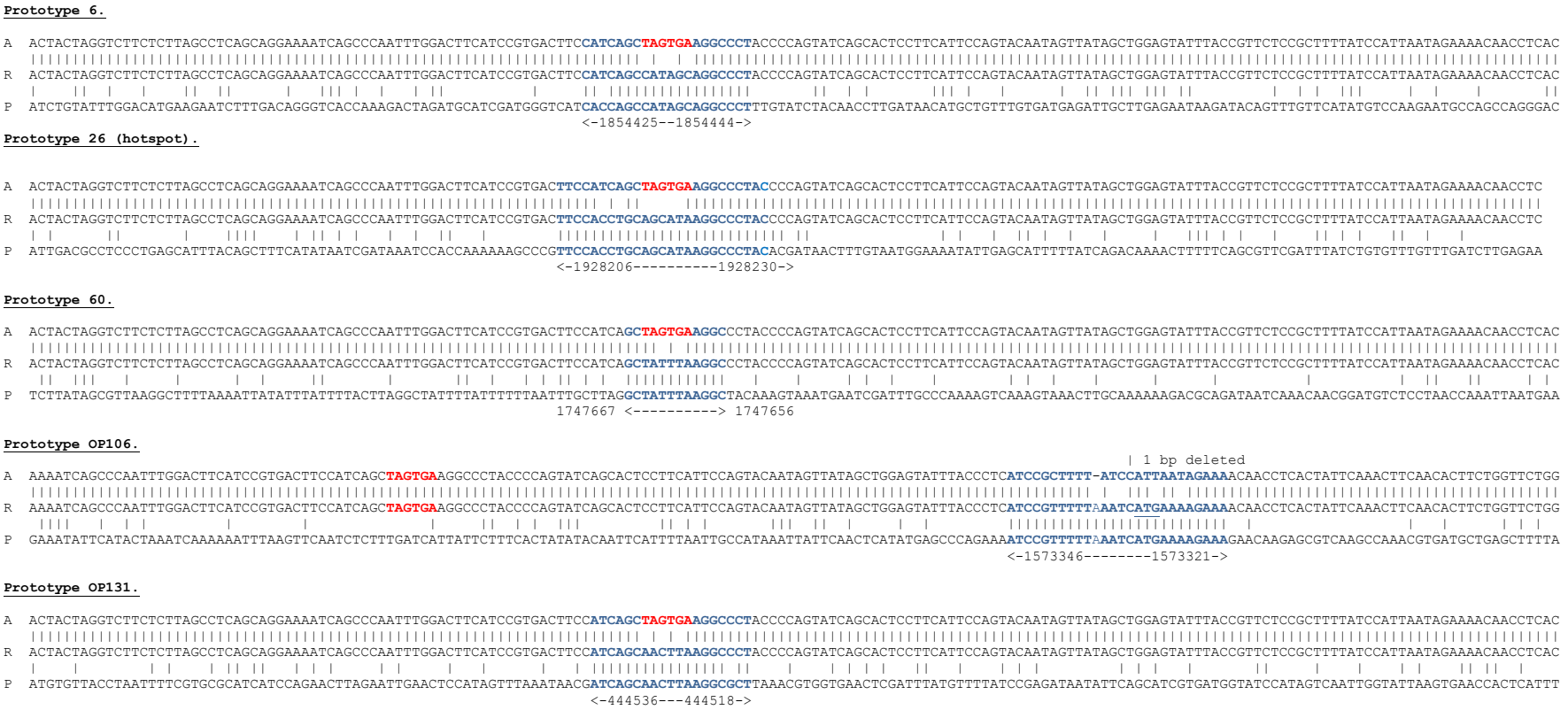

Figure 5: Overview of the class 1 SPIR prototypes: A single microhomology replacing the two stop codons by sense codons without deletions (exception: one nucleotide deleted in prototype OP106, and the stop codons are retained while a new start codon (underlined) is formed). See section 3.2.2.1. for more information on prototype 60. Alignments are shown in BLAST format. The patch numbers indicate the nucleotide positions in the published ADP1 sequence (accession number NC_005966). A: ancestral *hisC*::'ND5i' sequence. R: sequence of His$^+$ isolate. P: Sequence of patch used for illegitimate recombination. The consecutive stop codons are marked in red, and the patch alignment in blue. The prototypes named starting with OP were only observed in initial experiments by Overballe-Petersen et al., manuscript submitted (K. Harms, personal communication).

Figure 6: Overview of the class 2 SPIR prototypes: One microhomology upstream and one downstream of the two stop codons, with poorly aligned nucleotides and interspersed gaps, altogether resulting in small net deletions. This class of recombination can result in multiple amino acid substitutions (up to 26 in prototype OP59) in the resulting gene product. See Figure 5 for abbreviations and explanations.

Figure 7: Overview of the class 3 SPIR prototypes: One microhomology is located upstream and one downstream of the two stop codons (with a large number of nucleotides in the ancestral strand but few or no nucleotides in the patch), which results in larger deletions. See Figure 5 for abbreviations and explanations.

3.1.2.2.1. Origin of SPIR patches

To determine the source of the patches used in SPIR, the His$^+$ isolate sequence was aligned with the *Acinetobacter baylyi* ADP1 sequence (accession number NC_005966) using bl2seq. Out of 24 total SPIR mutants found, 18 of them had recombined with a patch located near the proposed terminus of replication (see Figure 8 for all chromosomal locations of the patches identified). All 7 SPIR mutants formed in AL1 (see section 3.2.) were recombined with a patch originating from the terminus of replication, suggesting that this is a hotspot for SPIR for strictly intragenomic recombination.

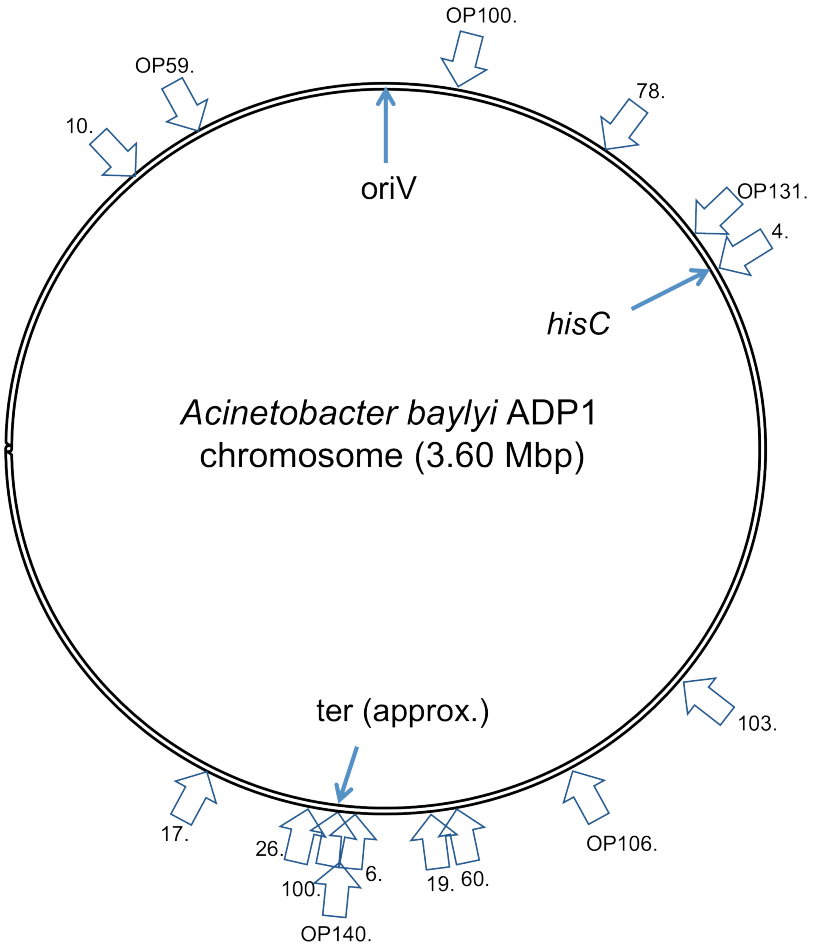

Figure 8: Patch locations. Each block arrow represents the location in the ADP1 genome where the different patches originate. The numbers next to the block arrows refer to the prototype each patch forms by SPIR (Fig. 5-7). Locations of *hisC*, terminus of replication and origin of replication are shown as simple arrows. Adapted and modified from K. Harms, unpublished.

3.2. Influence of natural transformation on SPIR

It is conceivable that the DNA used for the intragenomic recombination occurred fully or partially from dead cells releasing their genomic DNA into the culture, and active uptake of this DNA by the surviving naturally competent cells. To assess the influence of natural transformation on SPIR, the non-transformable strain AL1 [KOM218 Δ*comA*:: (*nptII sacB*)] was constructed as follows: KOM218 was transformed by linearized pKHNH2. Kanamycin-resistant and sucrose-sensitive colonies were isolated. The respective substitutions of *comA* by the *nptII sacB* genes were verified by PCR, and one isolate was named AL1.

3.2.1. Verification of transformation deficiency of AL1

To verify the non-transformability of AL1, the strain mixed with 100 ng/ml wildtype DNA (containing the *alkM*$^{+}$ allele that allows for growth on minimal medium with long-chain alkanes as carbon source) in a natural transformation experiment, washed and plated on histidine-supplemented M9 medium with hexadecane as sole carbon source provided in the gas phase. As positive control, KOM218 was treated correspondingly. After 72 hours incubation at 30°C, colonies were found for KOM218 but not for AL1, confirming that natural transformation is blocked in AL1.

3.2.2. His^{+} frequencies and characterization of AL1

The His^{+} frequency in AL1 was determined the same way as in KOM218. A total of 20 independent experiments were performed. The median frequency was determined to be 7.3×10^{-11} (Table 6), which is not different from that of KOM218. The AL1 His^{+} mutants were characterized using the same procedure as for KOM218. A total of 23 individual isolates were used for sequencing. The SPIR percentage was determined to be 30% (Table 6), which is the same as in KOM218.

3.2.2.1. Prototype 60

One His^+ isolate of AL1 (termed S60) had only two nucleotides exchanged, converting each stop codon into a sense codon (prototype 60, Figure 5), and thus resembles a mutant carrying two individual point mutations. The point mutation frequency has been determined in an *A. baylyi* Δ*recJ* Δ*exoX* strain (approximately 3×10^{-10}; K. Harms, personal communication). In turn, the likelihood of two independent point mutations occurring at this locus is about 10^{-19}, raising demand for alternative explanations. In order to assess a possible mutator phenotype, an assay screening for spontaneous resistance to chloramphenicol and ampicillin was devised. Mutation frequency (Table 9) was determined to be lower for S60 than that of KOM218 and AL1, which both are not mutators. Although bl2seq search did not identify a segment for illegitimate recombination even with relaxed parameters, a plausible patch (13 base pairs identical to the prototype 60 sequence) could be identified using the pDraw software (http://www.acaclone.com) that is located at the 1747656 – 1747668 position in the ADP1 genome that is close to the putative terminus of replication, suggesting an SPIR event. The ΔG^0_{min} was calculated for this patch and for all AL1 SPIR isolates (Table 8), and while the prototype 60 value was lowest for the class 1, it was higher than some of the bordering microhomologies of class 2 and 3.

Table 9: Spontaneous mutation frequency of the isolate S60 (3 individual colonies tested), KOM218 and AL1 to resistance against chloramphenicol and ampicillin.

Strain	Cm^r frequency	Amp^r frequency
S60	$7,4\times10^{-10}$	$7,7\times10^{-7}$
S60	$7,7\times10^{-9}$	$6,3\times10^{-7}$
S60	$4,3\times10^{-9}$	$6,0\times10^{-7}$
KOM218	$3,3\times10^{-7}$	$5,3\times10^{-7}$
AL1	$4,6\times10^{-8}$	$7,1\times10^{-7}$

3.3. Effect of the exonucleases RecJ and ExoX on SPIR

SPIR was detected in a strain lacking the single-strand-specific exonucleases RecJ and ExoX. To determine the effect of presence of ExoX and RecJ, the strain AL4

was constructed. AL4 is derived from the recombinational wildtype ADP1 strain JV28 with the ND5i segment inserted into the *hisC* gene and differs from KOM218 by having both *recJ*$^+$ and *exoX*$^+$. To determine the effect of RecJ alone, the strain AL6 was constructed. AL6 is derived from AL4 with *recJ* knocked out.

3.3.1. Construction of AL4 and AL6

The JV28 strain carrying the *trpE27* (tryptophan auxotrophy) allele was rendered *trpE*$^+$ by transformation by linearized pUC19-trpE5 plasmid DNA. An isolate growing on minimal medium was termed AL2. AL2 was naturally transformed by linearized pKHhisC20 plasmid DNA, substituting *hisC*$^+$ by *hisC*::(*nptII sacB*). Kanamycin-resistant isolates were confirmed to be auxotrophic and sucrose-sensitive, and the insertionally inactivated *hisC* allele was confirmed by PCR. One isolate was termed AL3. Next, AL3 was treated with linear pKHhisC25 plasmid DNA, and transformants were selected on sucrose and screened for kanamycin-sensitivity and by PCR for the *hisC*::'ND5i' allele. One isolate was termed AL4. In order to delete *recJ*, AL4 was transformed by linearized pEK2 DNA. Kanamycin-resistant (and sucrose-sensitive) isolates were isolated and verified by PCR to contain the D*recJ*::(*nptII sacB*) allele, and one isolate was named AL5. Strain AL5 was transformed by linear pEK3 DNA. Sucrose-resistant and kanamycin-sensitive isolates were screened by PCR. One resulting AL4 D*recJ* mutant was termed AL6.

3.3.2. His$^+$ frequencies of AL4 and AL6

His$^+$ frequencies for AL4 and AL6 were determined using the same procedure as for KOM218. A total of 20 independent experiments were done for AL4, and 19 experiments for AL6. The median His$^+$ frequency for AL4 was not calculable due to a majority of experiments yielding no His$^+$ colonies. The frequency of $<5.0\times10^{-11}$ was calculated by assuming that every experiment theoretically yielded one colony, and that the true frequency is at least lower than this. In AL6, the median His$^+$ frequency was 1.2×10^{-10} (Table 6).

3.3.3. Characterization of AL4 and AL6 His$^+$ isolates by sequencing

A total of 25 AL4 and 25 AL6 isolates were sequenced in the same manner as KOM218. 24 out of the AL4 sequences were deletion mutants, and a single SPIR mutant was found (Tables 6 and 7). The sequencing data of AL6 gave a similar result. In this strain, 22 deletion mutants and 1 SPIR mutant were found. Two of the AL6 His$^+$ isolates retained the *hisC*::'ND5i' sequence. The reason for the His$^+$ phenotype is unclear. Altogether, the proportion of the SPIR events is lower in the *recJ*$^+$ *exoX*$^+$ strain (4%) and in the Δ*recJ exoX*$^+$ strain (4%) than in the Δ*recJ* Δ*exoX* strains (32% and 30%, respectively), suggesting that the RecJ exonuclease is not, or only together with ExoX, the reason for the decreased SPIR frequency in wildtype cells.

3.4. Effect of addition of DNA

The experiments in this section were performed in collaboration with K. Harms. To determine whether exogenous DNA can influence SPIR formation, purified chromosomal DNA from *Bacillus subtilis* 168 (300 ng/ml) was added to standard mutation experiments of KOM218. In four independent assays, 68 His$^+$ colonies were detected. The median His$^+$ frequency was calculated as 6.1×10^{-10} although the number of experiments is too low to directly compare it to the results of Table 6. The *hisC* alleles from all His$^+$ isolates were determined by DNA sequencing. Altogether 14 independent deletion events and 19 independent SPIR events were identified (SPIR frequency 58%). In order to track illegitimate recombination events with the added DNA, bl2seq searches were performed as in section 3.1.2. and in addition with the GenBank sequences of two *B. subtilis* 168 genomes (accession numbers NC_000964 and NZ_CM000487). This led to the conclusions that five out of the 19 SPIR events were clearly, and one event possibly, generated by the added *B. subtilis* DNA that was horizontally acquired through natural transformation. The alignments are shown in Figure 9 and 10. This result shows that natural transformation in bacteria can lead to increased mutation frequency through SPIR.

Class 1 SPIR prototypes:

Figure 9: Overview of the SPIR prototypes formed with patches from *B. subtilis* 168 DNA (BS prefix), sorted by classes. For comparison, this Figure also shows the sole prototype obtained from natural transformation experiments (performed by K. Harms) with *B. subtilis* donor DNA using competent recipient *A. baylyi* KOM218 cells (personal communication, KH prefix). The patch numbers give the nucleotide position in the published GenBank sequence NC_000964. The sequences in GenBank NZ_CM000487 are identical although positions are different. In prototype BS45, a frameshift deletion of 121 bp occurred, and transcription *in frame* is possibly initiated by the upstream TTG (underlined) as start codon. See Figure 5 for abbreviations and explanations.

Prototype BS1.

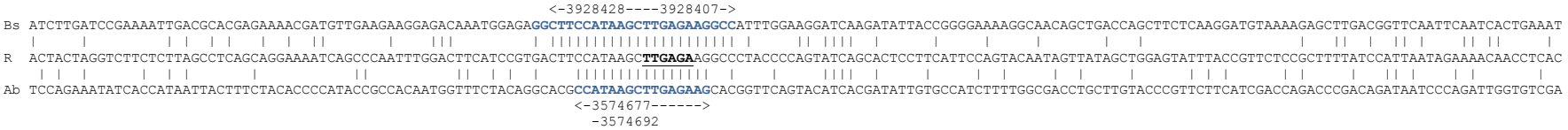

Figure 10: Alignment of SPIR prototype BS1 (centre, R) with possible patches from *B. subtilis* DNA (top, Bs, 24 bp) and *A. baylyi* (bottom, Ab, 16 bp). The patch positions refer to GenBank entries NC_000964 (Bs) and NC_5966 (Ab), respectively. The locations of the substituted stop codons of the ancestral *hisC*:: 'ND5i' allele are bold and underlined.

4. Discussion

In this thesis I have characterized SPIR, a novel mechanism for generating genetic diversity in *A. baylyi.* The results show that DNA patches as short as 12 base pairs can serve as substrates for IR events at microhomology-containing sites in the *A. baylyi* genome. These patches can either mediate deletions up to 168 bp, or introduce 2-8 SNPs alone or together with a smaller deletion (typically 3-9 bp). The frequency SPIR formations cannot be explained by classical mutation mechanisms, i.e. point mutations, since the cumulative mutations required per generation to mimic the His^+ isolates observed in this thesis would theoretically occur at extremely low frequencies. SPIR is also novel as a type of IR. IR up to now has been described either intracellularly as a process where DNA gyrase mediates joints between unrelated DNA ends (BIERNE *et al.* 1997; IKEDA *et al.* 2004), as joining of two molecules at microhomologous segments in the course of reactivation of stalled replication forks (BIERNE *et al.* 1997), as ligation of two different DNA molecules with and without microhomologies for gene amplification to occur (REAMS and NEIDLE 2004), or a process where partly homologous/heterologous DNA (acquired from the environment) is integrated by homology-facilitated illegitimate recombination process (HFIR and HFDIR) (DE VRIES and WACKERNAGEL 2002; HARMS *et al.* 2007; HULTER and WACKERNAGEL 2008a; PRUDHOMME *et al.* 2002). The reliance on microhomologies is what differentiates SPIR in this context. Whereas deletions, gene amplifications, HFDIR, and HFIR are observed both with and without microhomologies present, SPIR events occur strictly at microhomologies; SPIR events without presence of microhomology were not found in this study. This makes SPIR categorically different from Double Illegitimate Recombination (DIR), where no microhomology was present at all. SPIR is unique in that DNA from any source, i.e. heterologous DNA can be utilized, as long as the requirement of microhomology is fulfilled. It should be noted that the KOM218 construct only captures SPIR events *in frame.* This suggests that the frequencies reported here are underestimates, and that the true frequency of SPIR is approximately three times higher.

4.1. Point mutations as an alternative explanation for restored prototrophy

Spontaneous nucleotide exchanges in *A. baylyi* are detectable at frequencies about 1×10^{-10} per nucleotide in wildtype and 3×10^{-10} per nucleotide in a Δ*recJ* Δ*exoX* mutant (Overballe-

Petersen *et al.,* manuscript submitted). For a single mutant to obtain two mutations in the same locus (i.e. His prototrophy) the theoretical frequency would be approximately 3×10^{-10} × 3×10^{-10} = 9×10^{-20}. Thus the probability of a double point mutant to emerge from one generation to the next is very small (approximately eight orders of magnitude lower than SPIR). It is clear that for bacterial populations facing strong selective pressures where beneficial adaptation requires multiple mutations, SPIR may influence adaptation rates.

4.1.1. Prototype 60

The *hisC* allele of prototype 60 (see section 3.2.2.1. and Figure 5) contained a sequence resembling two point mutations restoring histidine prototrophy, with the two guanine residues in the consecutive stop codons substituted with thymine, inferring two transversions. The His^+ isolate was obtained in a non-transformable strain, excluding the substitutions to be a result of a transformation event. The probability of two point mutations occurring in this locus can be estimated as about 9×10^{-20} which is extremely unlikely to occur in the mutation experiments with about 2×10^{10} cells present per flask. Transversions generally occur less frequently than transitions, further reducing the probability of prototype 60 being a double point mutant (WANG *et al.* 2012). It is conceivable that a in a hypermutator strain independent point mutations occur at increased frequencies, but three individual prototype 60 colonies did not show a mutator phenotype either compared with non-mutator ancestral strains. By using the pDraw software, a suitable patch for SPIR was identified. The minimum free energy of hybridization (ΔG^0_{min}), which can be utilized to measure stability of hybridization between two complementary DNA single-strands ('stickyness'), was calculated for this patch. The free energy of hybridization was comparable to the other patches found in this thesis (see part 3. Results, Table 8) as well as to results of illegitimate recombination events found in other studies (HARMS *et al.* 2007; HULTER and WACKERNAGEL 2008a; HULTER and WACKERNAGEL 2008b). The patch source was located at the 1747656 – 1747668 position in the ADP1 genome, which is close to the proposed terminus of replication where all other patches for SPIR in the non-transformable strain originated. The free energy of hybridization, patch location and probability for a double point mutation occurring suggests that sample 60 gained prototrophy by an SPIR event, rather than individual point mutations. This conclusion suggests that SPIR may be a general and previously unrecognized explanation for multiple point mutation patterns.

4.2. Mechanism of action

It is likely that SPIR, like other microhomology-dependent recombination events, is not mediated by recombinases such as RecA although this has not been proven experimentally. Hybridization of single-strands in SPIR probably occurs at replication forks, during replication of the lagging strand (Figure 11). First, a segment of the donor strand hybridizes at a single (class 1) or two interrupted (class 2 and 3) microhomologous segments. Subsequently the ends of the donor strand at the hybridized part are processed. The chromosomal insertion may then simply occur by extension of the 3'-end of the strand by a DNA polymerase as has been proposed for short hybridizing DNA molecules (Dutra *et al*. 2007; Overballe-Petersen *et al*. unpublished). A DNA ligase seals the donor strand with the newly replicated strand, and then recombination is complete.

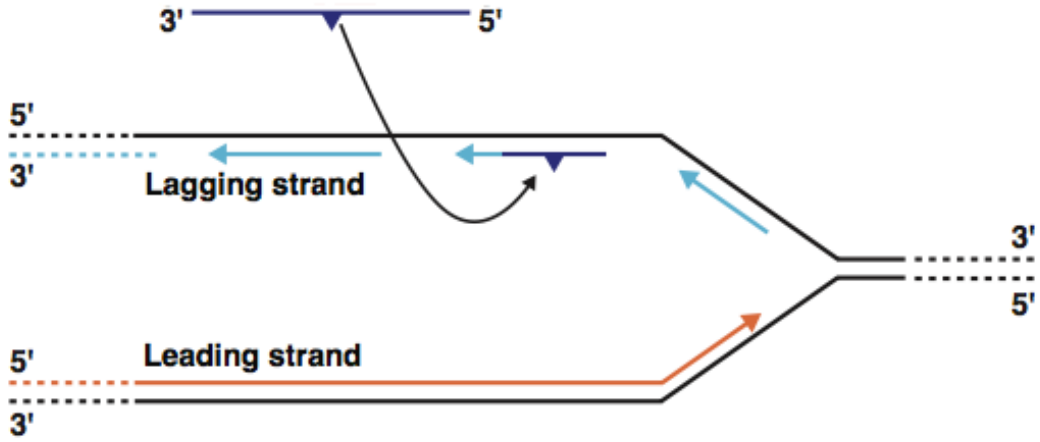

Figure 11: Proposed mechanism of SPIR. Heterologous DNA hybridizes in the lagging strand at microhomologous segments during replication, and is subsequently recognized as a primer by the replicative DNA polymerase. Adapted from Overballe-Petersen *et al*. manuscript submitted.

4.3. Patch sources

Most patches for SPIR, and all patches in the non-transformable strain (see Results, Figure 8), originated from the terminus of replication. The proposed mechanism of action suggests that the patching DNA molecule is single-stranded. Single-stranded DNA ends can be formed as a result of two replication forks colliding, which can lead to excess replication of DNA at the collision site (KAPLAN 2006). It is conceivable that this DNA when not or only partially

degraded is the source for SPIR events. The Tus protein is involved in terminating replication at the terminus, and without it, replication forks are more likely to collide. A way to investigate the role of replication termination in SPIR can be to knock out the *tus* gene in the non-transformable strain.

4.4. Influence of natural transformation

The experiments with addition of *Bacillus subtilis* DNA (Section 3.4.) show that SPIR ratio of the His^+ isolates is increased up to 58% and an approximately 15-fold increase in His^+ frequency. While some His^+ isolates formed prototypes with a patch originating from the *B. subtilis* genome, more interestingly addition of heterologous DNA showed an increase of SPIR with patches originating from the *A. baylyi* genome. Presence of single-stranded DNA following uptake initiates recombination attempts around the genome. These attempts typically do not lead to recombination due to lack of homology, but the duplex formations can still lead to or broken stalled replication forks, which subsequently during DNA repair can lead to generation of cleaved-off single-stranded DNA that can be used as patches. To test this hypothesis, further experiments can be designed with DNA damage-inducing agents. To cope with these agents the cell requires DNA repair, which produces single stranded DNA, which can ultimately lead to more SPIR events.

4.5. Role of single-strand-specific DNA exonucleases

The SPIR phenomenon was originally discovered in a genetic background where the single-strand-specific DNA exonucleases RecJ (described by LOVETT and KOLODNER 1989) and ExoX (VISWANATHAN and LOVETT 1999) were absent. By restoring these functions, the SPIR ratio dropped from 32% to 4%, suggesting that exonucleases play a role in limiting SPIR events. RecJ has been shown to affect IR events in different ways. In *E. coli*, absence of RecJ has been shown to remove illegitimate recombination of phage DNA at hotspots, suggesting that RecJ is necessary for this kind of IR (IKEDA *et al.* 2004). On the other hand, presence of RecJ has been shown to reduce occurrence of HFIR events in *A. baylyi* (HARMS *et al.* 2007), presumably by degrading non-hybridized DNA from the 5'-end after hybridization. In the strain with *recJ* knocked out but ExoX intact, SPIR frequency remained at 4%. This could be due to the inability of RecJ to degrade duplex DNA nucleotides (HAN *et al.* 2006), implying that the patch is safe from degradation by RecJ once it is hybridized. Consequently, the data

presented in this thesis suggests that ExoX is the key enzyme limiting SPIR in *A. baylyi*. ExoX degrades single-stranded DNA from 3'-5'. The putative mechanism of SPIR is dependent on single stranded DNA hybridizing in an open replication fork (Figure 11). How the DNA is trimmed down to a suitable patch size is however still not clear. Degradation of the donor DNA from 5'-3' is probably mediated by the exonuclease function of the polymerase in the lagging strand. The effect of ExoX could be that it binds the 3'-end of the donor fragment and degrades it in the 3'-5' direction before replication occurs. It should be noted that ExoX is able to degrade a 3'-single-strand end in duplex DNA, enabling it to process DNA at the patch even after hybridization has occurred (VISWANATHAN and LOVETT 1999).

4.6. Biological relevance

Data from the *recJ*$^{+}$ *exoX*$^{+}$ wildtype strain indicate that SPIR occurs at low but detectable frequencies. SPIR is relevant just like point mutations are relevant: it can introduce frameshifts and sense, silent and nonsense mutations. SPIR can also lead to deletions, which can be considered useful for removing redundant and non-functional DNA in a genome. It is conceivable that SPIR plays a greater (and yet unrecognized) role in protein evolution, generation of repeats, and genome stability.

4.6.1. Protein evolution

Protein evolution is a slow process where typically point mutations change the amino acid sequence in proteins, which potentially leads to change of specificity or function (PAL *et al.* 2006). In situations where multiple point mutations are necessary to impose a positive fitness effect, and the intermediate steps are detrimental, SPIR could potentially produce mutants that would overcome the such "adaptive hurdles", while point mutations would most likely not. One prototype found (OP59) shows a sequence where 26 sense codons are substituted. This demonstrates how dramatically SPIR can affect a protein in one single recombination event.

4.6.2. Generation of repeats

When a patch either originates from the cells own genome or is taken up from a dead clone of the same bacterial species, a sequence repeat is generated. Direct repeats can be

precursors for larger genomic rearrangements, such as deletions and amplifications/duplications. Amplifications have been shown to occur at microhomologies in *A. baylyi* (REAMS and NEIDLE 2004). SPIR could potentially create more microhomologies for such gene-amplifications to occur. Direct repeats are also involved in deletions by replication fork slippage (BIERNE *et al.* 1997), illustrating another potential role of SPIR in genome dynamics. Inverted repeats can lead to inversions (CRAIG 1985). It should also be noted that repeats generally cause genome instability and are desirable to avoid for bacteria (ROCHA *et al.* 1999).

4.7. Outlook

The effect of SPIR in a *recJ*$^{+}$ Δ*exoX* strain has not been demonstrated in this thesis, and should be investigated further to confirm the assumed effect of ExoX deficiency alone on SPIR. ExoX has been shown to not affect mismatch repair in *E. coli* (THOMS *et al.* 2008) and *A. baylyi* (Overballe-Petersen *et al.* manuscript submitted). It is however tempting to speculate that Δ*exoX* leads to a hypermutable phenotype, where increase in SPIR is the cause. While the increase in SPIR frequency is about 10-fold, the increase for potential of change in a single generation is comparable to the strong mutator phenotypes earlier described (MILLER 1996). In this context, looking at SPIR events in clinical strains could be an interesting follow-up. The effect of RecA on SPIR should be assessed as a control experiment. Experiments in a *tus* mutant or with DNA damaging agents should be done to test the hypothesis on recombination repair SPIR described in section 4.5.

4.8. Concluding remarks

The results obtained during the course of this thesis show that short patches of DNA can be subject to illegitimate recombination at short sequence-similarities called microhomologies. Here it is shown that SPIR either introduces a substitution of 2-8 base pairs alone or together with a 3-9 base pair deletion, or a larger deletion up to 168 base pairs. These SPIR events occur in both transformable and non-transformable strains. In transformation-deficient strains the patches observed in this thesis originate from the assumed terminus of replication, while in transformable strains in addition patches originating from all over the genome was utilized. Addition of *B. subtilis* DNA increases SPIR frequency approximately 15-fold. Patches originating from *B. subtilis* can be utilized for SPIR, but the observed SPIR %

increase also stems from patches native to the ADP1 genome. The first SPIR event was observed in a strain with the single-strand-specific exonucleases RecJ and ExoX. RecJ has been shown to have little effect on SPIR, but ExoX appears to reduce SPIR and has to be further investigated experimentally. SPIR can introduce multiple changes in a genome in a single event and in turn has the potential to accelerate the otherwise slow process of adaptive evolution.

5. References

ANDERSSON, D. I., and D. HUGHES, 2009 Gene amplification and adaptive evolution in bacteria. Annu Rev Genet **43:** 167-195.

BAQUERO, M. R., A. I. NILSSON, C. TURRIENTES MDEL, D. SANDVANG, J. C. GALAN *et al.*, 2004 Polymorphic mutation frequencies in Escherichia coli: emergence of weak mutators in clinical isolates. J Bacteriol **186:** 5538-5542.

BARBE, V., D. VALLENET, N. FONKNECHTEN, A. KREIMEYER, S. OZTAS *et al.*, 2004 Unique features revealed by the genome sequence of Acinetobacter sp. ADP1, a versatile and naturally transformation competent bacterium. Nucleic Acids Res **32:** 5766-5779.

BIERNE, H., S. D. EHRLICH and B. MICHEL, 1997 Deletions at stalled replication forks occur by two different pathways. EMBO J **16:** 3332-3340.

BURDETT, V., C. BAITINGER, M. VISWANATHAN, S. T. LOVETT and P. MODRICH, 2001 In vivo requirement for RecJ, ExoVII, ExoI, and ExoX in methyl-directed mismatch repair. Proc Natl Acad Sci U S A **98:** 6765-6770.

CARR, E. L., P. KAMPFER, B. K. PATEL, V. GURTLER and R. J. SEVIOUR, 2003 Seven novel species of Acinetobacter isolated from activated sludge. Int J Syst Evol Microbiol **53:** 953-963.

CHEN, I., P. J. CHRISTIE and D. DUBNAU, 2005 The ins and outs of DNA transfer in bacteria. Science **310:** 1456-1460.

CHEN, I., and D. DUBNAU, 2004 DNA uptake during bacterial transformation. Nat Rev Microbiol **2:** 241-249.

CLAVERYS, J. P., J. C. LEFEVRE and A. M. SICARD, 1980 Transformation of Streptococcus pneumoniae with S. pneumoniae-lambda phage hybrid DNA: induction of deletions. Proc Natl Acad Sci U S A **77:** 3534-3538.

CRAIG, N. L., 1985 Site-specific inversion: enhancers, recombination proteins, and mechanism. Cell **41:** 649-650.

DARWIN, C., 1859 *On the origin of species by means of natural selection*. J. Murray, London,.

DE VISSER, J. A., 2002 The fate of microbial mutators. Microbiology **148:** 1247-1252.

DE VRIES, J., and W. WACKERNAGEL, 2002 Integration of foreign DNA during natural transformation of Acinetobacter sp. by homology-facilitated illegitimate recombination. Proc Natl Acad Sci U S A **99:** 2094-2099.

DENAMUR, E., and I. MATIC, 2006 Evolution of mutation rates in bacteria. Mol Microbiol **60:** 820-827.

DRAKE, J. W., 1991 A constant rate of spontaneous mutation in DNA-based microbes. Proc Natl Acad Sci U S A **88:** 7160-7164.

DRAKE, J. W., B. CHARLESWORTH, D. CHARLESWORTH and J. F. CROW, 1998 Rates of spontaneous mutation. Genetics **148:** 1667-1686.

DUBEY, G. P., and S. BEN-YEHUDA, 2011 Intercellular nanotubes mediate bacterial communication. Cell **144:** 590-600.

DUTRA, B. E., V. A. SUTERA, JR. and S. T. LOVETT, 2007 RecA-independent recombination is efficient but limited by exonucleases. Proc Natl Acad Sci U S A **104:** 216-221.

ELENA, S. F., L. EKUNWE, N. HAJELA, S. A. ODEN and R. E. LENSKI, 1998 Distribution of fitness effects caused by random insertion mutations in Escherichia coli. Genetica **102-103:** 349-358.

FROST, L. S., R. LEPLAE, A. O. SUMMERS and A. TOUSSAINT, 2005 Mobile genetic elements: the agents of open source evolution. Nat Rev Microbiol **3:** 722-732.

FRYE, S. A., M. NILSEN, T. TONJUM and O. H. AMBUR, 2013 Dialects of the DNA uptake sequence in neisseriaceae. PLoS Genet **9:** e1003458.

GERRISH, P. J., and R. E. LENSKI, 1998 The fate of competing beneficial mutations in an asexual population. Genetica **102-103:** 127-144.

GOODGAL, S. H., 1982 DNA uptake in Haemophilus transformation. Annu Rev Genet **16:** 169-192.

GORE, J. M., F. A. RAN and L. N. ORNSTON, 2006 Deletion mutations caused by DNA strand slippage in Acinetobacter baylyi. Appl Environ Microbiol **72:** 5239-5245.

GRIFFITH, F., 1928 The Significance of Pneumococcal Types. J Hyg (Lond) **27:** 113-159.

GROHMANN, E., G. MUTH and M. ESPINOSA, 2003 Conjugative plasmid transfer in gram-positive bacteria. Microbiol Mol Biol Rev **67:** 277-301, table of contents.

HAN, E. S., D. L. COOPER, N. S. PERSKY, V. A. SUTERA, JR., R. D. WHITAKER *et al.*, 2006 RecJ exonuclease: substrates, products and interaction with SSB. Nucleic Acids Res **34:** 1084-1091.

HARMS, K., V. SCHON, E. KICKSTEIN and W. WACKERNAGEL, 2007 The RecJ DNase strongly suppresses genomic integration of short but not long foreign DNA fragments by homology-facilitated illegitimate recombination during transformation of Acinetobacter baylyi. Mol Microbiol **64:** 691-702.

HARMS, K., and W. WACKERNAGEL, 2008 The RecBCD and SbcCD DNases suppress homology-facilitated illegitimate recombination during natural transformation of Acinetobacter baylyi. Microbiology **154:** 2437-2445.

HULTER, N., and W. WACKERNAGEL, 2008a Double illegitimate recombination events integrate DNA segments through two different mechanisms during natural transformation of Acinetobacter baylyi. Mol Microbiol **67:** 984-995.

HULTER, N., and W. WACKERNAGEL, 2008b Frequent integration of short homologous DNA tracks during Acinetobacter baylyi transformation and influence of transcription and RecJ and SbcCD DNases. Microbiology **154:** 3676-3685.

IKEDA, H., K. SHIRAISHI and Y. OGATA, 2004 Illegitimate recombination mediated by double-strand break and end-joining in Escherichia coli. Adv Biophys **38:** 3-20.

JUNI, E., and A. JANIK, 1969 Transformation of Acinetobacter calco-aceticus (Bacterium anitratum). J Bacteriol **98:** 281-288.

KAPLAN, D. L., 2006 Replication termination: mechanism of polar arrest revealed. Curr Biol **16:** R684-686.

KICKSTEIN, E., K. HARMS and W. WACKERNAGEL, 2007 Deletions of recBCD or recD influence genetic transformation differently and are lethal together with a recJ deletion in Acinetobacter baylyi. Microbiology **153:** 2259-2270.

KOTRA, L. P., J. HADDAD and S. MOBASHERY, 2000 Aminoglycosides: perspectives on mechanisms of action and resistance and strategies to counter resistance. Antimicrob Agents Chemother **44:** 3249-3256.

KOTRA, L. P., and S. MOBASHERY, 1998 beta-Lactam antibiotics, beta-lactamases and bacterial resistance. Bulletin De L Institut Pasteur **96:** 139-150.

KOWALCZYKOWSKI, S. C., D. A. DIXON, A. K. EGGLESTON, S. D. LAUDER and W. M. REHRAUER, 1994 Biochemistry of homologous recombination in Escherichia coli. Microbiol Rev **58:** 401-465.

LECLERC, J. E., B. LI, W. L. PAYNE and T. A. CEBULA, 1996 High mutation frequencies among Escherichia coli and Salmonella pathogens. Science **274:** 1208-1211.

LORENZ, M. G., and W. WACKERNAGEL, 1994 Bacterial gene transfer by natural genetic transformation in the environment. Microbiol Rev **58:** 563-602.

LOVETT, S. T., and R. D. KOLODNER, 1989 Identification and purification of a single-stranded-DNA-specific exonuclease encoded by the recJ gene of Escherichia coli. Proc Natl Acad Sci U S A **86:** 2627-2631.

MAHILLON, J., and M. CHANDLER, 1998 Insertion sequences. Microbiol Mol Biol Rev **62:** 725-774.

MAJEWSKI, J., 2001 Sexual isolation in bacteria. FEMS Microbiol Lett **199:** 161-169.

MARSH, L., and G. C. WALKER, 1987 New phenotypes associated with mucAB: alteration of a MucA sequence homologous to the LexA cleavage site. J Bacteriol **169:** 1818-1823.

MEIER, P., and W. WACKERNAGEL, 2003 Mechanisms of homology-facilitated illegitimate recombination for foreign DNA acquisition in transformable Pseudomonas stutzeri. Mol Microbiol **48:** 1107-1118.

MILLER, J. H., 1996 Spontaneous mutators in bacteria: insights into pathways of mutagenesis and repair. Annu Rev Microbiol **50:** 625-643.

MIURA-MASUDA, A., and H. IKEDA, 1990 The DNA gyrase of Escherichia coli participates in the formation of a spontaneous deletion by recA-independent recombination in vivo. Mol Gen Genet **220:** 345-352.

MULLIS, K. B., 1990 The unusual origin of the polymerase chain reaction. Sci Am **262:** 56-61, 64-55.

Overballe-Petersen, S., Harms, K., Orlando, L., Mayar, J. V. M., Rasmussen S., Ponten, T. S, Brunak, S., Wackernagel, W., Inselmann, S., de Vries, J., Nielsen, R., Johnsen, P. J., Nielsen, K. M. and E. Willerslev, Manuscript submitted. Fragmented and Damaged DNA for Natural Transformation in Bacteria.

PAL, C., B. PAPP and M. J. LERCHER, 2006 An integrated view of protein evolution. Nat Rev Genet **7:** 337-348.
PALMEN, R., B. VOSMAN, P. BUIJSMAN, C. K. BREEK and K. J. HELLINGWERF, 1993 Physiological characterization of natural transformation in Acinetobacter calcoaceticus. J Gen Microbiol **139:** 295-305.
PERSKY, N. S., and S. T. LOVETT, 2008 Mechanisms of recombination: lessons from E. coli. Crit Rev Biochem Mol Biol **43:** 347-370.
PRUDHOMME, M., V. LIBANTE and J. P. CLAVERYS, 2002 Homologous recombination at the border: insertion-deletions and the trapping of foreign DNA in Streptococcus pneumoniae. Proc Natl Acad Sci U S A **99:** 2100-2105.
REAMS, A. B., and E. L. NEIDLE, 2003 Genome plasticity in Acinetobacter: new degradative capabilities acquired by the spontaneous amplification of large chromosomal segments. Mol Microbiol **47:** 1291-1304.
REAMS, A. B., and E. L. NEIDLE, 2004 Gene amplification involves site-specific short homology-independent illegitimate recombination in Acinetobacter sp. strain ADP1. J Mol Biol **338:** 643-656.
REDFIELD, R. J., 2001 Do bacteria have sex? Nat Rev Genet **2:** 634-639.
ROCHA, E. P., A. DANCHIN and A. VIARI, 1999 Functional and evolutionary roles of long repeats in prokaryotes. Res Microbiol **150:** 725-733.
RUHFEL, R. E., N. J. ROBILLARD and C. B. THORNE, 1984 Interspecies transduction of plasmids among Bacillus anthracis, B. cereus, and B. thuringiensis. J Bacteriol **157:** 708-711.
SALYERS, A. A., and C. F. AMABILE-CUEVAS, 1997 Why are antibiotic resistance genes so resistant to elimination? Antimicrob Agents Chemother **41:** 2321-2325.
SANGER, F., S. NICKLEN and A. R. COULSON, 1977 DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A **74:** 5463-5467.
SCHWARZ, S., C. KEHRENBERG, B. DOUBLET and A. CLOECKAERT, 2004 Molecular basis of bacterial resistance to chloramphenicol and florfenicol. FEMS Microbiol Rev **28:** 519-542.
SEEBERG, E., L. EIDE and M. BJORAS, 1995 The base excision repair pathway. Trends Biochem Sci **20:** 391-397.
TENAILLON, O., H. LE NAGARD, B. GODELLE and F. TADDEI, 2000 Mutators and sex in bacteria: conflict between adaptive strategies. Proc Natl Acad Sci U S A **97:** 10465-10470.
THOMS, B., I. BORCHERS and W. WACKERNAGEL, 2008 Effects of single-strand DNases ExoI, RecJ, ExoVII, and SbcCD on homologous recombination of recBCD+ strains of Escherichia coli and roles of SbcB15 and XonA2 ExoI mutant enzymes. J Bacteriol **190:** 179-192.
VAN HOEK, A. H., D. MEVIUS, B. GUERRA, P. MULLANY, A. P. ROBERTS *et al.*, 2011 Acquired antibiotic resistance genes: an overview. Front Microbiol **2:** 203.
VISWANATHAN, M., V. BURDETT, C. BAITINGER, P. MODRICH and S. T. LOVETT, 2001 Redundant exonuclease involvement in Escherichia coli methyl-directed mismatch repair. J Biol Chem **276:** 31053-31058.
VISWANATHAN, M., and S. T. LOVETT, 1999 Exonuclease X of Escherichia coli. A novel 3'-5' DNase and Dnaq superfamily member involved in DNA repair. J Biol Chem **274:** 30094-30100.
WANG, Z. Y., M. XIONG, L. Y. FU and H. Y. ZHANG, 2012 Oxidative DNA damage is important to the evolution of antibiotic resistance: evidence of mutation bias and its medicinal implications. J Biomol Struct Dyn.
Wetmur, J.G. (1996) Nucleic Acid Hybrids, Formation and Structure of. In *Encyclopedia of Molecular Medicine,* Vol. 4, Myers R. A. (ed.) New York: VCH Press, pp. 235-243.
YARON, S., G. L. KOLLING, L. SIMON and K. R. MATTHEWS, 2000 Vesicle-mediated transfer of virulence genes from Escherichia coli O157:H7 to other enteric bacteria. Appl Environ Microbiol **66:** 4414-4420.

6. Appendices

6.1. Appendix 1: *hisC*:: 'ND5i' insert sequence

>Acinetobacter baylyi ADP1 hisC::'ND5i' region
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