Acquisition of foreign DNA by natural transformation in *Acinetobacter baylyi*: quantitative estimates and molecular characterization of the introgression process

By

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ABSTRACT

Very little is known about introgression in bacteria. Introgression is the process where the genes of one species infiltrate the gene pool of another organism by subsequent backcross transformations of a hybrid with one of its parents. After the initial acquisition of foreign DNA, DNA from the newly made transformants is used as donor DNA in backcross transformations with the recipient. DNA is released to the environment after decomposing of dead cells, disrupting of cells or through excretion from living organisms. The extracellular DNA can be degraded after release, thus fragmented DNA can be taken up by bacteria.

One previous unpublished study investigated the effect of introgression of foreign unselected DNA. The donor DNA in this study was of high molecular weight (20 to 30 kilo bases (Kb)) and it was suggested that introgression in backcross transformation could be a mechanism by which unselected DNA was eliminated from the genome.

I wanted to study the effect of introgression when the foreign donor DNA was of low molecular weight (1000 to 4000 base pairs (bp)). We wanted to determine how fragmentation affects the speed at which unselected DNA from *Acinetobacter* sp. strain 16.4 was eliminated from the genome of *Acinetobacter baylyi* strain BD413 during the introgression process.

I have developed a method for optimal fragmentation of DNA to the desired size for this study. The DNA was fragmented by sonication, which gave an effective, gradual reduction in the fragment size of DNA. The size of the sonicated DNA was checked on an agarose gel and I found out that a gel fraction between 1000 to 4000 bp was the desired size for fragmented DNA. The DNA was extracted from the gel piece and used as low molecular weight donor DNA.

This method can be used to determine the effect of introgression when the foreign donor DNA is of low molecular weight (1000 to 4000 base pairs bp) and to get a better understanding for natural fragmentation of extracellular DNA in the environment.

TABLE OF CONTENTS

ABBREVIATIONS

bp	Base pairs
CFU	Colony forming unit
DNA	Deoxyribonucleic acid
EtBr	Ethidium Bromide
HGT	Horizontal gene transfer
Kb	Kilo bases
l	Liter
LB	Luria Broth
LBA	Luria Broth Agar
LBR	LB or LBA with rifampicin
LBRK	LB or LBA with rifampicin and kanamycin
LBSS	LB or LBA with streptomycin and spectinomycin
LBSSK	LB or LBA with streptomycin, spectinomycin and kanamycin
min	Minutes
ml	Milliliter
NaCl	Sodium chloride
ng	Nanogram
s	Seconds
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TF	Transformation frequency
TNTC	Too numerous to count
µl	Micro liter
µg	Micro gram

1. INTRODUCTION

1.1 Horizontal gene (HGT) transfer in bacteria

Horizontal gene transfer (HGT) is the genetic exchange between bacterial cells. The genetic material is transferred from one cell to another cell that is not its offspring, unlike in vertical gene transfer where the genetic material is transferred from mother to daughter cells. HGT can occur via three known mechanisms; conjugation, transduction and transformation. Conjugation is the transfer of mobile genetic elements, plasmids or transposons, from one bacterium to another by direct contact between donor and recipient cells.
Transduction is the transfer of genetic material from one bacterium to another by bacteriophages (viruses that infect bacteria). After the infection, the bacteriophage takes over the hosts DNA replication machinery and produce numerous new phages. Following lysis of the host cell, the phages can infect new host cells. Sometimes phages occasionally pack host DNA instead of phage DNA into viral packages and then inject this DNA into new host cells. The bacterial DNA can then integrate into the recipient DNA, causing horizontal gene transfer between bacteria (Thomas and Nielsen, 2005).
Natural transformation is the simplest of the three mechanisms of HGT. It requires free, extracellular DNA (plasmid or chromosomal) and a competent recipient bacterium (Lorenz and Wackernagel, 1994). Transformation was first demonstrated by Griffith (1928), and later it was demonstrated by Avery, MacLeod and McCarty (1944) that the transforming factor was DNA. Natural transformation involves uptake, incorporation and expression of the acquired DNA. More than 40 bacterial species are known to be naturally transformable (Lorenz and Wackernagel, 1994).

1.1.1 Molecular biology of natural transformation

In natural transformation the recipient is the bacterium which takes up the free DNA and the donor is the bacterium which the DNA comes from. The recipient has to be in a physiological state of competence (see next section) to be able take up free DNA. DNA becomes available for uptake in the environment by release from decomposing cells, disrupted cell or viral particles, or through excretion from living cells (Thomas and Nielsen, 2005). Natural transformation of a recipient cell with chromosomal donor DNA can be divided into four steps; development of competence, DNA uptake and binding, DNA translocation across the

inner and outer membrane and recombination/integration of the transforming DNA into the recipient chromosome (see the next sections).

Development of competence

Natural genetic competence is a genetically-encoded physiological state where the bacteria becomes capable of taking up naked/free/extracellular DNA (Chen and Dubnau, 2004; Dubnau, 1999; Lorenz and Wackernagel, 1994). Natural competence is a transient state in most naturally transformable bacteria (Lorenz and Wackernagel, 1994) and results from the growth of a bacterial culture under defined growth conditions, or it is, as in *Neisseria gonorrhoea* (Lorenz and Wackernagel, 1994; Thomas and Nielsen, 2005), constitutively expressed under all growth conditions (Dubnau, 1991) The evolution of the DNA uptake systems in bacteria can be accounted for by the use of incoming DNA as nutrition (Redfield, 2001), as templates for repair of DNA, or for genetic diversity by acquisition of potential useful genetic information such as antibiotic resistance (Chen and Dubnau, 2004). Competence has been found to involve approximately 20 to 50 proteins and approximately 1% of the validly described bacteria species have been found to be naturally transformable (Thomas and Nielsen, 2005).

DNA uptake and binding and translocation across the outer and inner membrane

Transport of extracellular DNA into the cytosolic compartment is a complex process (Chen and Dubnau, 2004). In gram-negative bacteria the DNA must cross the outer membrane, the cell wall and the cytoplasmic membrane in order to reach the cytoplasm. After exposition to competent bacteria the extracellular DNA binds non-covalently to sites present on the cell surface (Thomas and Nielsen, 2005). The number of binding sites ranges from 30 to 80 in *Acinetobacter baylyi* (Thomas and Nielsen, 2005). During translocation across the inner membrane, the DNA is converted from double stranded DNA (dsDNA) to single stranded DNA (ssDNA) (Averhoff and Graf, 2008; Thomas and Nielsen, 2005), the other strand is degraded during uptake. Sixteen competence genes has been identified in *Acinetobacter baylyi*; *comA*, *comEA, dprA, comP, comE, comF, comB, comC, pilD, pilC, pilB, comQ, comL, comM, comN, and comO* (see figure 1) (Averhoff and Graf, 2008). *comEA* is suggested to represent a soluble periplasmic protein and it may deliver bound DNA to *comA*. *comA* is most likely involved in transport of DNA through the cytoplasmic (inner) membrane. *dprA* is

thought to transport DNA through the cytoplasmic membrane and/or have a function in recombination. These three competence proteins are thought to be DNA translocator specific proteins. The rest of the competence proteins are thought to be type IV pili-related proteins. All of these mediate the contact with target surfaces. *comC* is involved in binding and transport of DNA and is co-located at the cells outer membrane with *comQ*, which translocates DNA through the outer membrane.

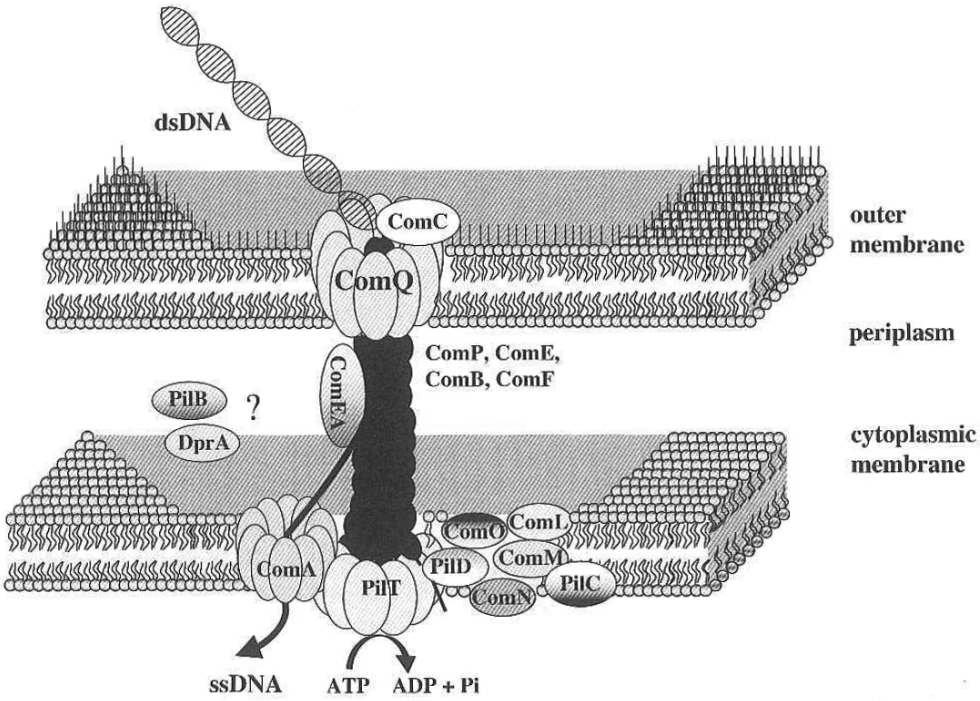

Figure 1. Model for uptake of DNA in *Acinetobacter baylyi* strain BD413. DNA is bound to ComC or directly to the ComQ proteins. Then the DNA bind to ComEA and is transported through the periplasmic space and peptidoglycan mediated by ComP, ComE, comB and ComF. Subsequent translocation across the cytoplasmic membrane is performed through a ComA channel (figure from Beate Averhoff and Iris Graf, 2008).

Recombination/integration of the transforming DNA into the recipient chromosome

After the single stranded chromosomal donor molecules have entered the cytoplasm they may invade the double stranded chromosome and become integrated in homologous regions of the recipient chromosome (Lorenz and Wackernagel, 1994). A recipient-donor heteroduplex is thought to be formed by homologous base-pairing between the donor strand and the corresponding recipient strand. For homologous recombination to occur the incoming DNA must contain regions of minimum 25 to 200 base pairs (bp) in length of similarity to the recipient genome (Thomas and Nielsen, 2005). These regions will initiate DNA pairing and

strand exchange. Some competent bacterial species, for example *Haemophilus influenza*, are selective in the DNA they bind and take up (they only bind and take up DNA from the same or closely related species), while most other species bind and take up DNA independently of its sequence (Lorenz and Wackernagel, 1994). The stability of the recipient-donor heteroduplex is dependent on the degree of sequence similarity between the donor and recipient DNA strain (Zawadzki et al., 1995). When the donor DNA is identical to the recipient chromosome, the integration of the donor will not result in a detectable genetic or phenotypic change (homogamic substitutive recombination, see figure 2A). When the donor DNA has some sequence dissimilarity compared to the recipient, part of the donor sequence can replace the recipient DNA (heterogamic substitutive recombination, see figure 2B). Another type of integration is called additive integration. It can occur when DNA sequences present only in the donor is flanked on both sides by sequences present in both donor and recipient (additive integration, see figure 2C). Additive integration can also occur when there is homology one side of the invading DNA strand and random microhomology (3-8 bp) at the other (de Vires and Wackernagel, 2002). This type of integration is called homology-facilitated illegitimate recombination (HFIR) (see figure 2D).

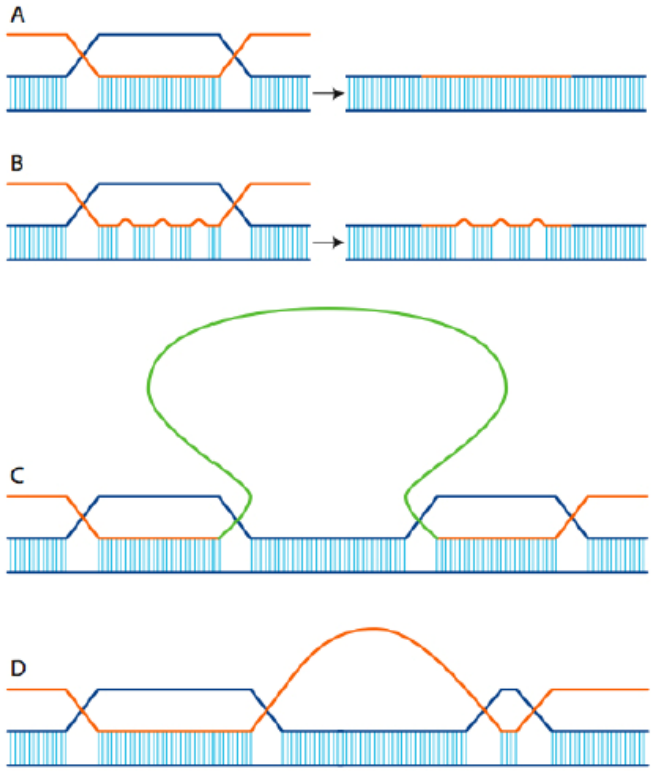

Figure 2. Various recombination types in bacteria. (A) Homogamic substitutive recombination in which donor (orange line) and recipient (blue line) DNA are sequence-identical, leading to no change in the recipient. (B) Heterogamic substitutive recombination occur when some mismatches exist between donor and recipient. (C) Additive integration occurs when a unique DNA sequence (green line) in the donor is integrated into the recipient through homogamic/heterogamic recombination on each side of the unique DNA sequence. (D) HFIR, integration of donor DNA occurs by a stretch of homology on one side and random microhomology on the other side (figure by Jessica Louise Ray, 2007).

1.2 The genus *Acinetobacter* and the model organism *Acinetobacter baylyi* strain BD413

The genus *Acinetobacter* is a group of highly versatile, gram-negative bacteria which are ubiquitous in nature (Barbe et al., 2004). They are highly capable for adaptation and are found in different habitats, including water, soil, sewage, living organisms and as a part of the normal microflora on human skin. Several strains of the genus are capable of utilizing a variety of compounds (including hydrocarbons, aliphatic alcohols, glycols, carbohydrates and amino acids) as sole carbon and energy source. Bacteria of this group are strictly aerobic, oxidase-negative, immobile and do not form spores. In the microscope they look like cocci (in stationary phase) or like short bacilli, and they often appear in pairs or as longer chains (see figure 3) (Barbe et al., 2004).

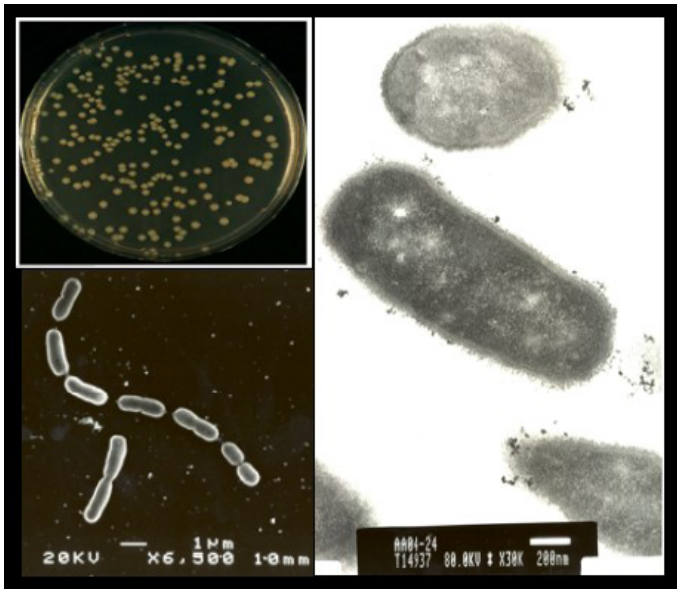

Figure 3. *Acinetobacter baylyi* strain BD413. Clockwise from top left: 2-day-old colonies on a LB plate, transmission electron micrograph, and scanning electron micrograph. Photograph and micrographs by Kåre M. Nielsen.

Acinetobacter baylyi is a naturally transformable gram-negative gamma-proteobacterium. It is found in aquatic and soil environments. *A. baylyi* is able to grow overnight in both rich and minimal salts media. It can grow slowly at room temperature, but has an optimal growth between 30 and 37ºC (Metzgar et al., 2004). *A. baylyi* is non-pathogenic to humans, but *A. baumannii*, another species within the genus *Acinetobacter*, is an opportunistic human pathogen and can cause serious infections in humans, especially in immuno-compromised patients (Tomaras et al., 2008). *A. baylyi* has a small genome composed of a single circular chromosome composed of 3 598 621 base pairs (bp) (Barbe et al., 2004).

In 1911, a Dutch microbiologist, Beijerinck, described an organism that was isolated from soil (Dijkshoorn and Nemec, 2008). The organism was isolated by enrichment cultivation on a calcium acetate-mineral medium. He named the organism *Micrococcus calco-aceticus*. In 1954 Brisou and Prèvot created the genus *Acinetobacter* (Barbe et al., 2004).

A. baylyi strain BD4 was originally isolated from soil by Taylor and Juni in 1960 (Barbe et al., 2004). The isolated strain was of an encapsulated gram-negative cocci which used 2,3-butanediol as the sole carbon source (Taylor and Juni, 1961). They called the strain BD4 for ButaneDiol. Mutagenesis of strain BD4 yielded an unencapsulated, nonclumping mutant called BD413 (Juni and Janik, 1969). *A. baylyi* strain BD413 is the European name, while *A. baylyi* strain ADP1 is the American name for the same bacterium (renamed in 1985 in the laboratory of Nicholas Ornston at Yale university (Barbe et al., 2004)).

1.3 Introgression

Introgression is the process where the genes of one species infiltrate the gene pool of another organism by subsequent backcross transformations of a hybrid with one of its parents. After the initial acquisition of foreign DNA, the DNA of the newly made transformants is used as donor DNA in repeated backcross transformations with one of its parents as recipient. In the initial transformation between a donor, containing a *npt*II gene (resistance gene), and a recipient, the *npt*II gene will be integrated into the recipient's chromosome as well as an unknown length of heterologous donor DNA. It is expected that there will be a gradual elimination of heterologous flanking DNA sequence in the transformants during the introgression process (see figure 4).

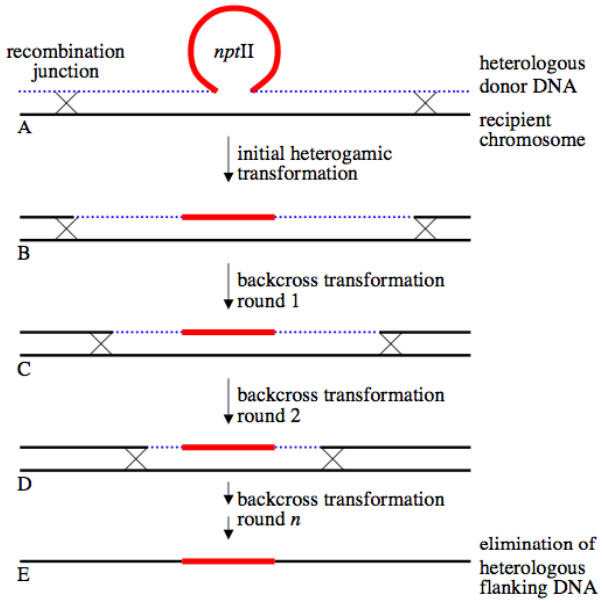

Figure 4. Figure of the hypothetical gradual elimination of heterologous flanking DNA sequence in transformants during the introgression process. Stippled, blue line, donor DNA; solid black line, recipient chromosome; solid red line, *nptII* gene; X symbols, recombination sites. (A) Initial heterogamic transformation between *npt*II-tagged donor DNA and kanamycin sensitive recipient chromosome. This results in integration of the *npt*II gene and also some heterologous donor DNA in the recipient chromosome. (B) DNA from resulting transformants in the initial transformation is used as donor DNA in transformation with the original recipient. This results in elimination of a certain amount of donor chromosomal DNA in the transformants. (C-D) When the process is repeated, further elimination of donor chromosomal DNA occur in the transformants, until (E) donor chromosomal DNA is completely eliminated and only *npt*II is left (figure by Jessica Louise Ray, 2007).

There are, to my knowledge, no published studies on introgression in bacteria. An unpublished introgression study (Ray et al., unpublished) examined the molecular events that occur during introgression of the *npt*II gene with different parental origins at unique loci in

naturally transformable bacteria. 18 isolates (generated from 3 *Acinetobacter* spp. strains) were used as donor DNA in the first backcross transformation. Chromosomal DNA from *Acinetobacter* spp./ *A. baylyi* strain BD413 hybrid transformants, containing the *npt*II gene, were used as donor DNA in subsequent backcross transformations with *A. baylyi* strain BD413. The same experiments were performed using the mutator strain *A. baylyi* ADP7021 as recipient. Hybrid DNA from the 18 isolates transformed recipient BD413 or ADP7021 cells at homologous frequencies (~10^{-3} transformants recipient^{-1}) already during the first backcross transformation. Subsequent rounds of backcross transformations were performed with transformant DNA as donor. This demonstrated stability of restored homogamic frequencies for up to 10 observed backcross generations. For one of the donor isolates, a closer inspection of 10 generations of hybrid transformants in the BD413 line, revealed some loss of heterologous transfer signal linked to the transferred *npt*II gene. There was no significant difference in transformation frequency between the mutator strain and the wild type strain in the introgression study.

1.4 Experimental design of this study

In this introgression study we wanted to use fragmented chromosomal bacterial donor DNA and compare the results with the results from the previously performed unpublished introgression study (Ray et al., unpublished). After the initial acquisition of foreign DNA, the DNA of the newly made transformants were used as donor DNA in repeated backcross transformations with the wild type or the mutator strain as recipient (see figure 5).

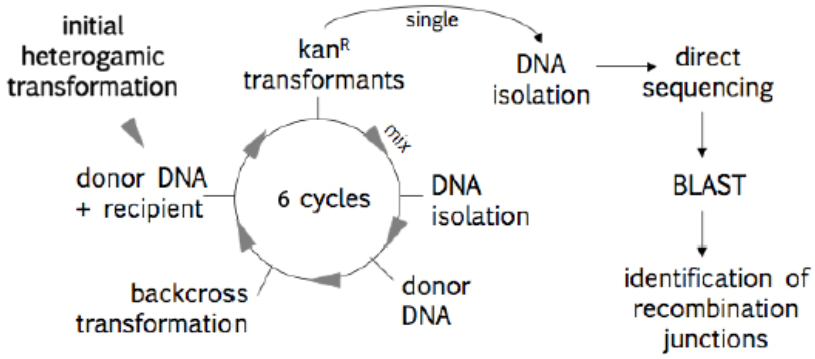

Figure 5. Figure illustrating the experimental design for the introgression study. From left: a heterologous donor (*A.* sp. strain 16.4) was used as donor to transform *A. baylyi* strain BD413 or *A. baylyi* strain ADP7021 (both are resistant to rifampicin) in heterogamic transformation. Then the resulting transformants, which were resistant to rifampicin and kanamycin (kan^R), due to transfer of the *npt*II gene, were selected. DNA was isolated from the transformants and the isolated genomic DNA was used in subsequent back transformations. For identification of recombination joints, the *npt*II gene insertion and the flanking regions, DNA was isolated from single transformants and sequenced (figure by Jessica Louise Ray, 2007).

The *A. baylyi* strain used as recipient in this study is isogenic to BD413, but resistant to rifampicin. It is a spontaneous rifampicin-resistant mutant (Nielsen et al., 1997). It is transformable with both plasmid and chromosomal DNA and it does not discriminate between heterologous and homologous DNA when it comes to uptake (Nielsen et al., 1997).

The mutator strain *A. baylyi* strain ADP7021 (Young and Ornston, 2001) was also used as recipient. *A. baylyi* strain ADP7021 (ΔmutS6::Ω) is a derivative of *A. baylyi* strain BD413, but has a mutS deletion. It is expected that it will have a higher transformation frequency then the wild type due to the defect (inactivation of mutS) in the mismatch repair system. *A. baylyi* strain ADP7021 is resistant to spectinomycin and streptomycin.

In this study, *A.* species 16.4 (Ray, 2007) was used a donor DNA. It has a *npt*II gene (neomycin phosphotransferase II), encoding kanamycin resistance, inserted in a *filA* like gene in the genome. *A.* sp. 62A1.4 has a 24.4% sequence divergence from *A. baylyi* strain BD413 (approximately every fourth base is different). The initial transformation between a *npt*II-tagged donor (*A.* sp. 62A1.4) and a recipient that is resistant to rifampicin (*A. baylyi* strain BD413) will result in transformants who have the *nptII* gene integrated, as well as an unknown length of heterologous donor DNA. It is expected that there will be a gradual elimination of heterologous flanking DNA sequence in the transformants during the

introgression process (see figure 4). The resulting transformants were resistant to both rifampicin and kanamycin.

In this study, six backcross transformations were performed with one isolate generated from the *Acinetobacter* sp. 62A1.4 as donor DNA and *Acinetobacter baylyi* strain BD413 as recipient. There was also performed one backcross transformation with *Acinetobacter baylyi* strain ADP7021 as recipient. Contrary to an earlier performed study, the donor DNA was fragmented in my study before it was used in the transformation assays. The results were compared to the earlier performed study (Ray et al., unpublished) with unfragmented donor DNA.

Before the study with fragmented DNA could be accomplished, we had to develop a method to fragment total DNA. We wanted to test the effect of decreasing size of donor DNA on transformation frequency in BD413. The total DNA should be fragmented for so long that the fragment size was around 3000 bp, which included both the *npt*II gene (1000 bp) and some flanking regions, and that the filter transformation resulted in a small number of transformants.

1.5 Aims and objectives

The aim of this study is to determine how fragmented DNA affects the introgression process of chromosomal DNA in *A. baylyi* compared to the use of unfragmented DNA.

Objectives:

- To determine if DNA fragmentation affects the speed at which heterologous flanking DNA is eliminated during successive rounds of back transformation using the wild type strain BD413 or the mutator strain ADP7021 as recipients, and highly fragmented DNA of one nptII tagged isolates of 16.4 (*A.* sp. strain 62A1.4) as donor DNA.
- To develop a method for optimal fragmentation of chromosomal bacterial DNA.
- To estimate the transformation frequency of fragmented DNA of ADP1200-2 and 16.4 in filter transformations with *A. baylyi* strain BD413.
- To collect transformant colonies, isolate DNA from these colonies and use it as donor DNA in subsequent backcross transformations.
- To perform DNA sequencing of the flanking DNA in resulting transformants to identify possible cross-over junctions.
- To create a transformant strain archive for further use in the introgression studies.

2. MATERIALS AND METHODS

2.1 Bacterial strains

Acinetobacter baylyi strain BD413 is used as recipient cells and total DNA extracted from *A. baylyi* ADP1200-2 and *Acinetobacter* species 62A1 are used as donor DNA. *A. baylyi* strain ADP1200-2 (Johnsen, unpublished) and ADP1200 (Kok et al., 1999) are isogenic except from a 227 bp deletion rendering the kanamycin resistance gene (*npt*II) inactive in ADP1200. DNA from ADP1200-2 is homologous to BD413 and is used as a positive control in the filter transformations. *A.* sp. 62A1 (Ray, 2007) is called 16.4 in our strain collection, were 16 indicates the strain 62A1 and 4 indicates the location of the *npt*II gene on the chromosome. The *npt*II gene in 16.4 is inserted in a *filA* like gene in the donor *A.* sp. 62A1 strain (Ray, 2007). *A.* sp. 62A1 has a 24.4% sequence divergence from BD413 (approximately every fourth base is different). *A. baylyi* BD413 is resistant against the antibiotic rifampicin, the strains ADP1200-2 and 62A1 are resistant against the antibiotic kanamycin.

The mutator strain *Acinetobacter baylyi* strain ADP7021 (Young and Ornston, 2001) is a derivative of *Acinetobacter baylyi* strain BD413. It has a mutS deletion (ΔmutS6::Ω) and is resistant to spectinomycin and streptomycin. It is also used as recipient cells with 62A1 as donor DNA.

2.2 Bacterial growth media

Luria Broth (LB) was made by dissolving 25 g Luria Broth (Invitrogen, Germany/Fluka Sigma-Aldrich, Germany) in 1 L distilled, boiled water and autoclaving it for 20 min at 121ºC. **LB agar (LBA)** was made by dissolving 25 g LB (Fluka Sigma-Aldrich, Germany) and 12 g Agar-Agar (Merck, Germany) in 1 L distilled, boiled water and autoclaving it for 20 min at 121ºC. **LBK^{50}** was made by adding kanamycin (50 mg/L) (Fluka Sigma-Aldrich, Germany) to1 L LB, **LBR^{50}** was made by adding rifampicin (50 mg/L) (Fluka Sigma-Aldrich, Germany) to 1 L LB and **LBRK$^{50/50}$** was made by adding rifampicin (50 mg of each/L) to 1 L LBK^{50}. **LBSS$^{50/50}$** was made by adding streptomycin and spectinomycin (10 mg/L and 50 mg/L) to 1 L LB and **LBSSK$^{50/50/50}$** was made by kanamycin (50 mg/L) to 1 L LBSS$^{50/50}$.

2.3 Glycerol stock of competent cells

A glycerol stock of competent cells of BD413 was made by adding one colony of BD413 competent cells to 3 ml LBR^{50} and incubating it overnight with shaking (200 rpm). The next day the ON-culture was added to 100 ml LBR^{50} in a 1000 ml Erlenmeyer flask and incubated with shaking (120 rpm) for 3 hours and 50 minutes. The absorbance was measured using the Ultraspec 2000, UV/Visible Spectrophotometer, and should be around 0.600 A. The suspension was centrifuged for 10 minutes, at 4000 rpm at 4 ºC. The supernatant was discarded and the pellet was dissolved in 15% glycerol/LB to a CFU (colony forming unit) of $1*10^9$ cells/ml. The solution of competent cells of BD413 was transferred to Eppendorf tubes (1 ml in each tube) and stored at -75ºC.

2.4 Isolation of Genomic DNA from bacteria

Genomic DNA was isolated using the Genomoc-tip 100/G (QIAGEN, Germany) protocol in the QIAGEN Genomic DNA Handbook (Qiagen, 2001).

Day 1. An overnight culture (ON culture) was made by inoculating 3 ml of LB media (with the suitable antibiotic) with one colony of bacteria, and incubating it overnight with shaking.

Day 2. The ON culture was diluted 1:10 in the same media as the ON culture and incubated with shaking (120 rpm) in a 50 ml Falcon tube or a 100 ml Erlenmeyer flask for 5-7 hours. The lids on the Falcon tubes were left loose to avoid development of anaerobic conditions. The tubes were centrifuged in an Eppendorf 5810R bench top cooling centrifuge for 10 minutes at 4000 rpm and 4ºC. The supernatants were discarded. The pellets, about 2-4 mm thick, were stored overnight at - 20 ºC.

Day 3. Lysozyme (100 mg/ml) (Sigma, Germany), RNaseA (100 mg/ml) (Sigma, Germany), proteinase K (10 mg/ml) (Sigma, Germany) and the tubes with the pellets were taken out from the freezer and put on the bench until they thawed. Buffer QC and QF were preheated to 60ºC. 7 µl RNAseA was added to 3.5 ml Buffer B1 for each prep and mixed in a Falcon tube by turning the tube up-side-down two times. The bacteria pellet was resuspended in 3.5 ml of Buffer B1/RNAaseA by vortexing at top speed for 10-15 seconds. 80 µl lysozyme and 100 µl

proteinase K were added, followed by vortexing for 5 seconds and incubating at 37ºC (water bath) for 60-90 minutes.
If the lysates were transparent 1.2 ml Buffer B2 was added, followed by vortexing for 2-3 seconds and incubating at 50ºC (water bath) for 30 minutes. The QIAGEN Genomic-tip 100/G column was equilibrated with 4 ml Buffer QBT. Each sample solution was vortexed for 10 seconds and immediately applied to the QIAGEN Genomic-tip 100/G. The QIAGEN Genomic-tip 100/G was washed with 2 x 7.5 ml Buffer QC. The Genomic-tip was placed over a new 50 ml Falcon tube and the DNA was eluted with 5 ml Buffer QC. The DNA was precipitated by adding 3.5 ml isopropanol and inverting the tube 20 times. The DNA precipitate was collected by using a plastic inoculation loop (10 µl), transferred into a new Eppendorf-Tube and washed with 500 µl 100 % etanol. The DNA was transferred to a new Eppendorf-Tube and set to air-dry before it was resuspended in 100 µl dH_2O and incubated overnight at 4ºC to dissolve completely.

Day 4. The Eppendorf tubes were vortexed for 5 seconds and then centrifuged for 10 minutes at 13000 rpm and 4ºC in the Biofresco cooling centrifuge. The DNA containing supernatant was transferred to a new tube and the concentration was measured with the NanoDrop® ND-1000 spectrophotometer (NanoDrop Tecnologies Inc, USA). The DNA samples were stored at -20ºC. Typically DNA yield was 1000-2000 ng/µl. The ratio of sample absorbance at 260 and 280 nm (260/280) was determined to assess the purity of DNA.

2.5 Fragmentation of DNA

DNA was fragmented and used as donor DNA filter transformations.

2.5.1 Fragmentation of DNA by nebulization

Nebulization (to reduce the liquid into small particles / to atomize the liquid into a fine spray) of DNA was done to fragment DNA, following the manufactures recommendation. The nebulizer (Invitrogen, USA) (Figure 6) was assembled by unscrewing the blue top and slipping the vinyl tubing over the atomizer. The top was screwed back on and the nebulizer was connected to a nitrogen tank. A regulator was used to regulate the pressure. 25-50 µg DNA was added to 750 µl Shearing Buffer (5 ml 1M Tris HCl, 50 ml 0,001 EDTA, 50 ml glycerol and 395 ml dH_2O, pH adjusted to 8 with 2M NaOH) and pipeted into the bottom of

the nebulizer. The capped nebulizer was placed in an ice bucket to keep the DNA cold. The DNA was sheared for 10 seconds to 10 minutes at 10 psi. The size of the fragmented DNA was determined on a 1% agarose gel.

700 ul of the sheared DNA or what was left in the nebulizer was transferred to an Eppendorf tube and 80 µl 3 M sodium acetate (pH 5.2), 4 µl 20 mg/ml glycogen and 700 µl 100% isopropanol was added. The solution was mixed well and put in the freezer (-20ºC) for 30 minutes or overnight. Then the tube was centrifuged at 12000 rpm for 15 minutes at 4ºC. The supernatant was removed by using water suction and the pellet was washed with 800 µl of icecold 80% ethanol. Then the tube was centrifuged for 5 minutes and the ethanol was removed. The tube was centrifuged again for 1 minute and all traces of ethanol were removed by using water suction. The pellet was further dried by using a heating block at 37ºC. The DNA was resuspended in 70 µl dH_2O and the concentration was measured using NanoDrop® spectrophotometer.

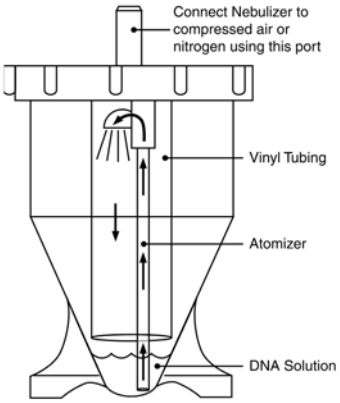

Figure 6. The nebulizer used for fragmentation of DNA (Nebulizer Introduction) (figure taken from www.tools.invitrogen.com/content/sfs/manuals/nebulizer_man.pdf).

2.5.2 Fragmentation of DNA by sonication

The DNA was fragmented by sonication (ultrasound), following the manufactures recommendation. The Bioruptor UCD-200 (Diagenode, Belgium) was set on “High”. The tank was filled 1 cm of chrushed ice and filled up to the indicated level with cold water (4ºC). The samples were placed in the tank and sonicated for 5 seconds to 60 minutes. The size of the fragmented DNA was determined on a 1% or a 1.5% agarose gel. Since the Bioruptor UCD-200 have a 30 sec “ON” and 30 sec “OFF” cycle, the total time for 60 minutes sonication was 2 hours. The water and the ice were changed every 15 minutes to keep the conditions for sonication as constant as possible.

2.6 Agarose gel preparation and gel electrophoresis

Gel electrophoresis is used to separate DNA fragments by size. DNA, which is negatively charged, migrates from negative to positive potential and the smallest molecules migrate longest. The agarose gel was made by heating the agarose solution in a microwave until the agarose melted. It was cooled down to 40 ºC by holding it under cold water and then either ethidiumbromide (EtBr) (Sigma-Aldrich, Germany) (10 mg/ml) was added or the gel was stained in SYBR (SYBR®Safe DNA gel stain in 1 x TAE, Invitrogen, Germany) for 40 minutes with agitation after the gel was run. The gel solution was poured into a gel rack and a comb was inserted to cast the wells. After the gel had become solid (about 30 minutes), the comb was removed and the gel with the rack were put into a gelchamber with TBE or TAE buffer. The gel was completely covered with buffer and the ladder 1 Kb Plus DNA ladder (Invitrogen, Germany) or 1 Kb DNA ladder (AB gene, UK) and the samples were applied to the wells. 6 x T or 40 % sucrose are used as loading buffer in the samples. The gel was "run" at 90 V for 1 hour.

The 1 to 1.5 % agarose gel contained 0.5 to 0.75 g LE agarose (SeaKem®, USA) or low melting agarose (prod., land), 50 ml 1 x TBE or TAE buffer and 5 µl EtBr.

10 x TAE buffer was made by 108 g tris base, 55 g boric acid, 40 ml 0.5 M EDTA (pH 8.0) and adding dH_2O to a total of 1 l. The buffer was diluted 10 x in dH_2O before use.

50 x TAE buffer was made by 242 g tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA (pH 8.0) and adding dH_2O, to a total of 1 l. The buffer was diluted 50 x in dH_2O before use.

2.6.1 Extraction of DNA fragments from agarose gels

DNA was extracted from the agarose gel using the QIAquick gel extraction kit protocol in the QIAquick® Spin Handbook (Qiagen, 2006). A gel piece with DNA fragments between 1000 bp and 4000 bp was excised from the agarose gel with a scalpel. EtBr was removed from the gel slices by laying them in dH_2O for 40 minutes before they were weighed in a 15 ml Falcon tube. Then 3 volumes of solubilizing and binding buffer QG were added to 1 volume of gel. The tube was incubated at 50ºC until the gel slices had dissolved. The tube was turned up-side-down a few times to help dissolving the gel. Then a QIAquick spin column was placed in a provided 2 ml collection tube. 750 µl of the samples were applied to the column and centrifuged for 1 min at 13000 rpm. The flow-through was discarded and the process repeated

until the entire sample had been centrifuged. Then 0.5 ml of buffer QG was added to remove all traces of agarose and the sample was centrifuged for 1 min. 0.75 ml of buffer PE was added two times to wash out impurities and the column was centrifuged for 1 min. The flow-through was discarded and the column centrifuged for additional 1 min. The DNA was eluted in a clean Eppendorf tube by adding 30 µl dH_2O to the center of the membrane. The column was left for 1 min before it was centrifuged for 1 min. The DNA concentrations of the samples were measured using the NanoDrop® spectrophotometer.

2.7 Filter transformation

25 µl or 65 µl DNA (approximately 500 ng/µl) was transferred to each Eppendorftube. A sterile filter was placed asymmetrical on a LB plate. The filters used were 0.22 µm thick, white GSWP, 47 mm in diameter (Millipore, UK) and they were autoclaved in distilled water for 20 minutes by 121ºC at 200 kPa. A glass rod was brushed over the filter to remove air bubbles. 65 µl 0.9% NaCl was spread out on one filter as filter sterility control. 100 µl 0.9 % NaCl was spread out on a LB plate as NaCl sterility control. The competent cells (1 ml) were taken out of the freezer (-70ºC), put into the Biopico centrifuge and spun at room temperature for 10 minutes at 4000 rpm. The supernatant was removed and the cells were dissolved in 810 µl 0.9 % NaCL. 100 µl or 250 µl of the competent cells was transferred to each Eppendorftube with DNA and mixed by pippetting up and down two times. 100 µl from each Eppendorftube was spread out on three filters each (three parallels). When only 100 µl competent cells were used, everything from the tube was spread out on one filter. All the plates were wrapped in plastic and incubated at 33ºC for 20-24 hours. The first three filters were always 25 or 100 µl NaCl mixed with competent cells (negative control).
25 µl DNA (~ 500ng/µl) and 100 µl competent cells (*A. baylyi* strain BD413 or strain ADP7021) was used per filter in filter transformation with strain 16.4-16.4.6 as donor DNA, when the DNA was extracted from an agarose gel.
65 µl DNA (~ 500ng/µl) and 250 µl competent cells (*A. baylyi* strain BD413) was used per 3 filter in filter transformation with *A. baylyi* strain ADP1200-2 and 16.4 as donor DNA to determine the transformation frequency.
The next day the filters were transferred to a new Falcon tube, containing 2 ml or 4 ml 0.9 % NaCl. The filters were washed by using a pipette or by vortexing for 10 seconds. Either undiluted or different dilutions from each test were plated out on different LB plates with or without selection (selection: $LBRK^{50/50}$ or $LBSSK^{50/50/50}$, no selction: LBR^{50} or $LBSS^{50/50}$).

When 25 µl donor DNA was used, 100 µl of the undiluted test was plated out on one plate with selection to check the growth of the competent cells and on one plate without selection to check the growth and to determine the number of the transformants, also 500 µl and 1300 µl (the rest) were plated out on plates with selection. When 65 µl donor DNA was used 1 ml from each tube was diluted to 10^{-7} with 0.9 % NaCl and 100 µl of the different dilutions from each test were plated out on plates with and without selection, 3 parallels of each. All the plates were wrapped in plastic and incubated at 33ºC for 2 days, before counting. To calculate the transformation frequency of ADP1200-2 and 16.4 the total number of transformants and the total number of recipients was calculated and used in the following formula:
Transformation frequency = (# Transformants)/ (# Recipients)
Transformants = number of colonies (colony forming unit (CFU)) on plates with selection x dilution factor
Recipients = CFU on plates without selection x dilution factor

2.8 Direct DNA sequencing of genomic DNA from bacteria

DNA sequencing was used to determine the insertion site of the *npt*II gene and the flanking DNA regions.

The amount of template in each reaction was between 3 and 5 ng. Two different reaction mixes were used:
Reaction mix 1: 4 µl BigDye version 3.1 (ABI, USA), 2 µl DNA (400-600 ng/µl), 2 µl DNA sequencing buffer (5x) (ABI, USA) and 4 µl primer (see table 1).
Reaction mix 2: 8 µl BigDye version 3.1, 8 µl DNA (400-600 ng/µl) and 4 µl primer (see table 1).

Table 1. Primers used in the sequencing of 16.4 and five single colonies of 16.4.0, 16.4.5 and 16.4.10.

Primer	Recognition sequences 5'- to -3' direction	Supplier
Apr 25	TGTTGGATTTTGGGGAGAAG	Operon
Apr 27	CGTAATGCTCCACTTGCAGA	Operon
LP853	TTCTCCTTCATTACAGAAACGG	Operon
LP1046	TTGAAGGATCAGATCACGCATCTTCCCGA	Sigma Genosys
RP152	TTGAATATGGCTCATAACACCCC	Sigma Genosys
RP255	TTGTCGCACCTGATTGCCC	Operon
16.4 FW24	TGGTGATTTACAGCCAACTCAA	Operon
16.4 RV20	TAAATGCTGGTCCACAGGTCTT	Operon

Operon (Germany), Sigma Genosys (Germany)

The cycle sequencing reaction was run on a PTC-200 Peltier Thermal Cycler (MJ Reasearch) programmed as follows: initial denaturation at 95º for 5 minutes, followed by 99 cycles of: denaturation at 95º for 30 seconds, annealing at 55º for 10 seconds and elongation at 60º for 4 minutes, then storage at 4º C.

The products from the cycle sequencing were precipitated and sequenced at the DNA-sequencing laboratory at the University of Tromsø, using the 3130x1 Genetic Analyzer (Applied Biosystems, USA). The resulting sequences were edited using Sequencher^{TM} 4.2.2 (Gene Codes, USA).

3. RESULTS

3.1 DNA isolation

Total genomic DNA was isolated, fragmented and used as donor DNA in filter transformations. DNA was isolated from *A. baylyi* strain ADP1200-2 and *A.* sp. strain 16.4 and from the transformants of the different generations of *A.* sp. 16.4 in *A. baylyi* strain BD413 and *A. baylyi* strain ADP7021. The DNA pellet was resuspended in 100 µl dH_2O and the concentration was measured. Then the DNA was diluted in dH_2O to an approximate concentration of 0.5 µg/µl before further use. Tables 2 to 4 give an overview of the average yield of isolated DNA, it was generally between 150 to 200 µg.

Table 2. Yield from DNA-isolation of *A. baylyi* strain ADP1200-2 and *A.* sp. 16.4.

Sample	µg/µl	Total yield in µg	260/280*
Strain ADP1200-2	2.0	199.9	1.89
Strain 16.4	2.1	211.4	1.89

* The ratio of sample absorbance at 260 and 280 nm (260/280) assess the purity of DNA

Table 3. Yield from DNA-isolation from various *Acinetobacter* strains.

Strain[a]	µg/µl	Total yield in µg	260/280
16.4	2.0	202.8	1.88
16.4.0 tot. pop.	1.9	185.1	1.88
S16.4.0.1	2.5	248.6	1.88
S16.4.0.2	2.2	224.1	1.80
S16.4.0.3	2.0	191.8	1.86
S16.4.0.4	2.8	279.2	1.85
S16.4.0.5	2.4	239.6	1.86
16.4.1 tot. pop.	2.1	209.0	1.90
16.4.2 tot. pop.	2.1	206.5	1.89
16.4.3 tot. pop.	1.8	178.9	1.89
16.4.4 tot. pop.	2.1	213.1	1.89
16.4.5 tot. pop.	2.0	196.9	1.87
S16.4.5.1	2.3	228.1	1.88
S16.4.5.2	1.7	171.7	1.89
S16.4.5.3	1.6	159.4	1.90
S16.4.5.4	1.8	183.7	1.88
S16.4.5.5	1.9	187.7	1.89

[a] tot. pop. = total population, all the colonies from one plate were picked and used, S= single colony, one colony from one plate was picked and used.

Table 4. Yield from DNA-isolation from various mutator strains of *Acinetobacter*.

Strain[a]	µg/µl	Total yield in µg	260/280
16.4	2.3	235.0	1.88
MS16.4.0.1	0.9	93.7	1.89
MS16.4.0.2	2.0	201.1	1.89
MS16.4.0.3	2.0	199.8	1.89
MS16.4.0.4	1.8	181.3	1.89
MS16.4.0.5	1.8	178.0	1.89
MS16.4.1.1	1.7	168.6	1.89
MS16.4.1.2	1.6	155.2	1.90
MS16.4.1.3	1.2	119.7	1.88
MS16.4.1.4	1.8	183.0	1.88
MS16.4.1.5	1.6	160.1	1.89

[a] MS= mutator single colony, one colony from one plate was picked and used.

3.2 Method for optimal fragmentation of DNA

As already described in the experimental settings, we wanted to fragment chromosomal bacterial DNA to around 1000 to 3000 base pairs (bp) and test the effect on transformation frequency (TF) in *A. baylyi* strain BD413. The donor DNA was fragmented and used to find out if fragmentation affects the TF and the speed at which heterologous flanking DNA is eliminated during successive rounds of back transformations. Two methods were used to fragmentate the DNA, nebulization and sonication.

3.2.1 Fragmentation of DNA by nebulization

In order to develop a method for fragmenting total genomic DNA, we nebulized the DNA from *A. baylyi* ADP1200-2 with 10 psi for up to 10 minutes. Around 50 µg DNA and 750 ml Shearing Buffer was nebulized for 0 to 600 seconds (10 minutes). There was a gradual reduction in fragment size with increasing nebulization and after 10 minutes most of the fragments were around 1500 bp (fig.7). Figure 7 shows nebulized DNA after it has been precipitated (five nebulizations). The precipitated, nebulized DNA was used as donor DNA in filter transformation with *A. baylyi* strain BD413 (see table 1 in Appendix). The yield of precipitated, nebulized DNA is shown in table 5.

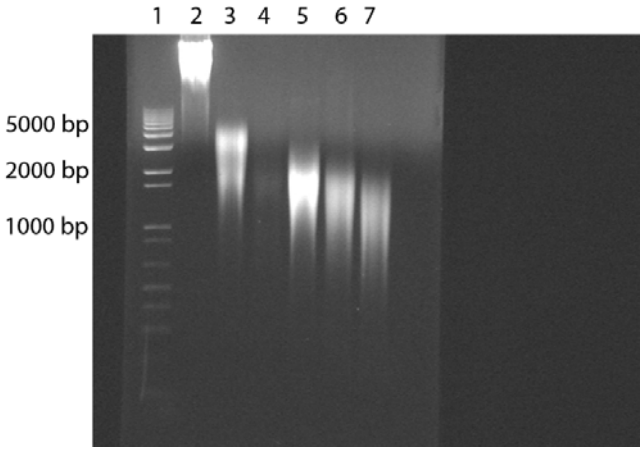

Figure 7. Isolated DNA of *A. baylyi* strain ADP1200-2 nebulized for 0 to 600 seconds (10 minutes), then precipitated. Lanes: 1: 1 Kb Plus DNA ladder (Invitrogen, Germany), 2: 0 s, 3: 40 s, 4: 120 s, 5: 240 s, 6: 360 s, 7: 600 s.

Table 5. Yield from precipitated, nebulized DNA of *A. baylyi* strain ADP1200-2 for 0 to 600 seconds.

Nebulized ADP1200-2	µg/µl	Total yield in µg	260/280
40 s	0.4	33.6	1.88
120s	0.6	44.2	1.85
240s	0.5	43.8	1.87
360s	0.4	33.6	1.84
600s	0.4	28.2	1.83

Table 6 and figure 8 shows the transformation frequencies (TF) of nebulized DNA of *A. baylyi* strain ADP1200-2. The TF decreased from $3.9*10^{-3}$ for unnebulized DNA to $2.2*10^{-5}$ for DNA nebulized for 600 seconds (10 minutes).

Table 6. Average transformation frequency of *A. baylyi* strain ADP1200-2 nebulized for 0 to 600 seconds in filter transformation with *A. baylyi* strain BD413.

ADP1200-2	Average transformation frequency	Standard deviation
Unnebulized	3,0E-03	6,6E-04
Nebulized for 40s	1,8E-05	9,1E-07
Nebulized for 120s	7,0E-07	1,5E-07
Nebulized for 240s	2,3E-05	6,2E-06
Nebulized for 360s	8,9E-07	9,7E-08
Nebulized for 600s	2,2E-05	3,8E-06

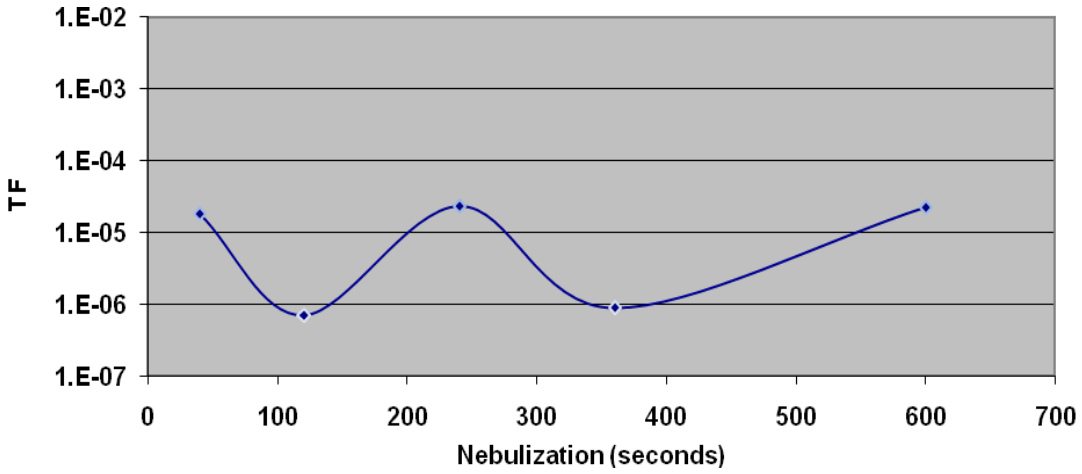

Figure 8. Transformation frequency of isolated DNA of *A. baylyi* strain ADP1200-2, nebulized for 0 to 600 seconds (0 to 10 minutes), in *A. baylyi* strain BD413.

After 600 seconds (10 minutes) of nebulization there was a great loss of liquid (DNA and Shearing buffer), there was only 400 µl liquid left in the nebulizer. Even though the size of the DNA fragments was around 1500 bp it still gave transformants (see table 1 in Appendix). We wanted to se how long we could nebulize the DNA before it did not give any transformants. But when nebulized for longer than 600 seconds (10 minutes) there was not enough liquid left to use further in the filter transformation.

3.2.2 Fragmentation of DNA by sonication

Because we lost too much DNA during nebulization due to technical limitations with the nebulizer, we fragmented the DNA with ultrasound (sonication). We sonicated the DNA from *A. baylyi* strain ADP1200-2 up to 3600 seconds (60 minutes). The fragment size of the sonicated DNA decreased with increasing sonication (fig. 9 and 10). After 600 seconds of sonication the fragment size was around 650 bp (fig. 9).

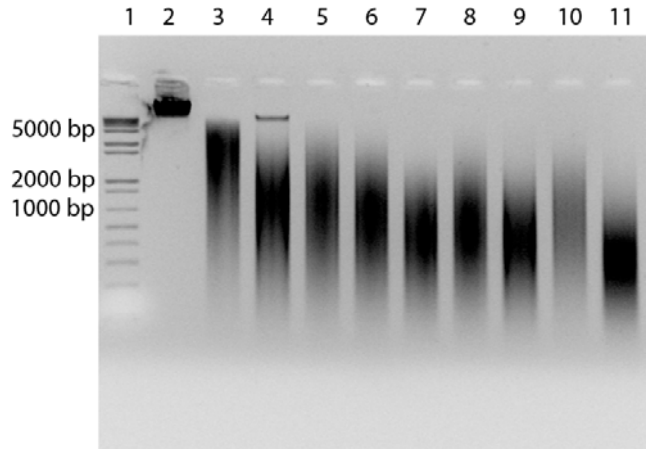

Figure 9. Isolated DNA of *A. baylyi* strain ADP1200-2 sonicated for 0 to 600 seconds (0 to 10 minutes). Lanes: 1: 1 Kb Plus DNA ladder (Invitrogen, Germany), 2: 0 s, 3: 10 s, 4: 30 s, 5: 60 s, 6: 120 s, 7: 180 s, 8: 240 s, 9: 300 s, 10: 360 s, 11: 600 s (image is inverted).

Because the average size of DNA was 650 bp after 600 seconds of sonication and the *npt*II gene is ~1220 bp, it was a surprise to detect a similar TF as for DNA sonicated for 10 seconds. We wanted to find out how long we could sonicate the DNA before the TF was reduced to zero. We sonicated DNA from *A. baylyi* strain ADP1200-2 for 1200 seconds and up to 3600 seconds (20 to 60 minutes) (fig. 10) to see how this affected the TF.

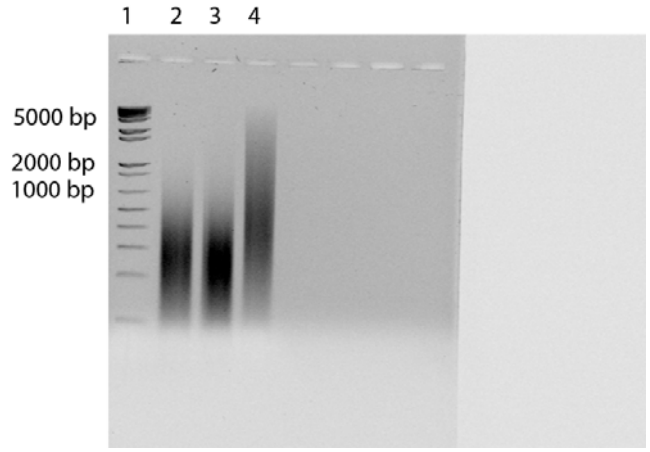

Figure 10. Isolated DNA of *A. baylyi* strain ADP1200-2 sonicated for 1200 to 3600 seconds (20 to 60 minutes). Lanes: 1: 1 Kb Plus DNA ladder (Invitrogen, Germany), 2: 1200 s, 3: 1800 s, 4: 3600 s (image is inverted).

Table 7 and figure 11 shows the TF of sonicated DNA of *A. baylyi* strain ADP1200-2. The TF decreased from $3.9*10^{-3}$ for unfragmentated DNA to $3.7*10^{-6}$ for DNA sonicated for 3600 seconds (60 minutes). Sonication of DNA turned out to be a good method to fragmentate DNA.

Table 7. Average transformationfrequency of *A. baylyi* strain ADP1200-2 sonicated for 0 to 3600 seconds (0 to 60 minutes).

Strain ADP1200-2	Average transformation frequency	Standard deviation
Unsonicated	3,9E-03	5,9E-04
Sonicated for 10s	7,3E-06	2,4E-06
Sonicated for 60s	3,5E-06	1,9E-06
Sonicated for 120s	2,4E-06	2,5E-07
Sonicated for 180s	3,6E-06	8,7E-07
Sonicated for 600s	2,5E-06	5,3E-07
Sonicated for 1200s	1,2E-07	3,4E-08
Sonicated for 1800s	1,3E-07	2,7E-08
Sonicated for 3600s	3,7E-06	7,8E-07

0.9% NaCl was used as negative control, there was no growth on the LBRK$^{50/50}$ plates (LB plates with rifampicin and kanamycin) with 0.9% NaCl and competent cells because *A. baylyi* strain BD413 is not resistant against kanamycin, unsonicated *A. baylyi* strain ADP1200-2 was used as positive control.

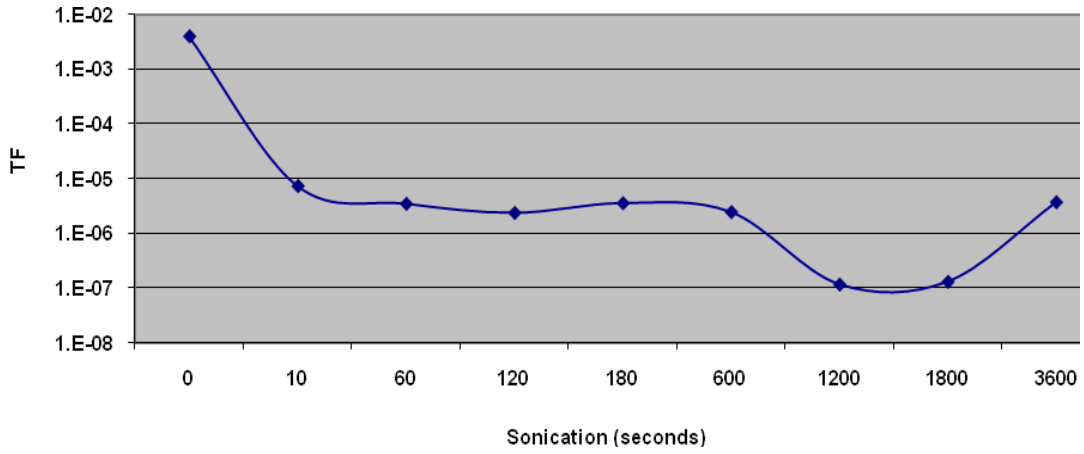

Figure 11. Transformation frequency of isolated DNA of *A. baylyi* strain ADP 1200-2, sonicated for 0 to 3600 seconds (0 to 60 minutes), in *A. baylyi* strain BD413. (A) shows the whole graph, (B) shows the lower part of the graph from 10 seconds to 3600 seconds of sonication.

Because homologous donor DNA (*A. baylyi* strain ADP1200-2) sonicated for up to 3600 seconds (1 hour), still gave transformants, we wanted to find out how sonication of heterologous donor DNA (*A.* sp. strain 16.4) affected the transformation. We sonicated the DNA from *A.* sp. strain 16.4 up to 600 seconds (10 minutes). The fragment size of the sonicated DNA decreased with increasing sonication (fig.12 to 13). After 600 seconds (10 minutes) of sonication the fragment size was around 500 bp. We tried to use sonicated DNA

for up to 600 seconds (10 minutes) as donor DNA, but only DNA sonicated for 5 and 10 seconds gave transformants (see table 3 in Appendix).

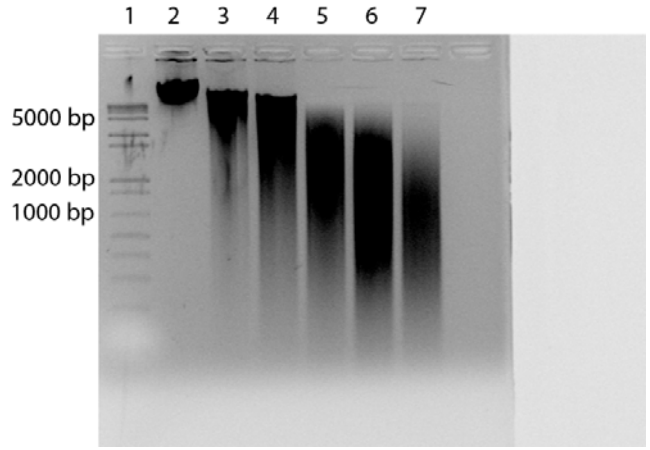

Figure 12. Isolated DNA of *A.* sp. strain 16.4 sonicated for 0 to 60 seconds. Lanes: 1: 1 Kb Plus DNA ladder (Invitrogen, Germany), 2: 0 s, 3: 5 s, 4: 10 s, 5: 20 s, 6: 30 s, 7: 60 s (image is inverted).

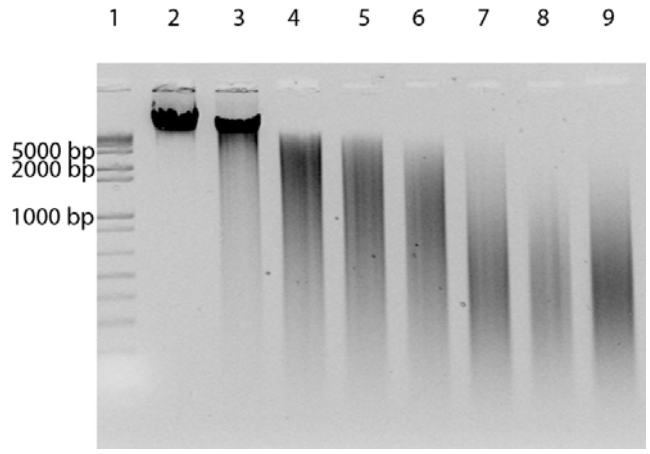

Figure 13. Isolated DNA of *A.* sp. strain 16.4 sonicated for 0 to 600 seconds (0 to 10 minutes). Lanes: 1: 1 Kb Plus DNA ladder (Invitrogen, Germany), 2: 0 s, 3: 10 s, 4: 20 s, 5: 30 s, 6: 60 s, 7: 120 s, 8: 180 s, 9: 600 s (image is inverted).

As shown in table 8 and figure 14, the TF only decreases slightly from 2.0 $*10^{-9}$ for unfragmentated DNA to $1.2*10^{-9}$ for DNA sonicated for 10 seconds. DNA of *A.* sp. strain 16.4 sonicated for up to 600 seconds (10 minutes) was used as donor DNA in filter tansformation with *A. baylyi* strain BD413, but only DNA sonicated for up to 10 seconds gave transformants.

Table 8. Average transformation frequency of isolated DNA from *A.* sp. strain 16.4 sonicated for 0 to 20 seconds.

Strain 16.4	Average transformation frequency	Standard deviation
Positive control	4,2E-03	3,9E-03
Unsonicated	2,0E-09	5,2E-10
Sonicated for 5s	5,2E-10	7,3E-10
Sonicated for 10s	1,2E-09	7,3E-10
Sonicated for 20s	0	-

0.9% NaCl was used as negative control, there was no growth on the LBRK$^{50/50}$ plates with 0.9% NaCl and competent cells because *A. baylyi* strain BD413 is not resistant against kanamycin, unsonicated *A.* sp. strain ADP1200-2 was used as positive control. *A.* sp. strain 16.4 was sonicated for 0 to 600 seconds, but the TF was 0 when the DNA was sonicated for more than 10 seconds.

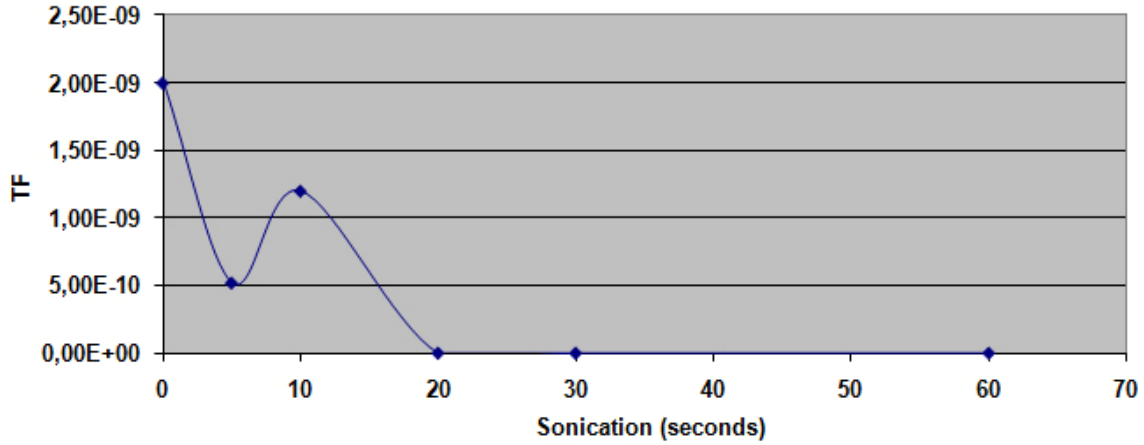

Figure 14. Transformation frequency of isolated DNA of *A.* sp. strain 16.4, sonicated for 0-60 seconds, in *A. baylyi* strain BD413. (The DNA was sonicated for 0 to 600 seconds, but this graph only shows 0 to 60 seconds of sonication. The TF was 0 when the DNA was sonicated for more than 10 seconds.)

3.2.3 Extraction of DNA-fractions from agarose gel

To avoid that unfragmented DNA contributed to the transformation when the DNA was fragmented by sonication, it was decided to make sure that only fragmented DNA was used as donor DNA. This was done by cutting out a piece form the agarose gel that only contained fragmented DNA and extract DNA from it. The extracted DNA could then be used as donor DNA in filter transformation. To test this method it was necessary to find out from which size range of fragmented DNA that contributed to the transformation. A 1% agarose gel was made and 10 µl sonicated *A.baylyi* strain ADP1200-2 DNA was applied in each well. Then after the gel was run, three gel pieces were cut out. The first gel fraction was up to 1500 bp, the second was from 1500 to 3000 bp and the third was from 3000 to 10000 bp. DNA was extracted from the three gel pieces and used as donor DNA in filter transformation with *A. baylyi* strain

BD413. There were no resulting transformants. The reason for this was probably the small volume of DNA applied to the wells and the low yield of extracted DNA as shown in table 9.

Table 9. Yield from DNA extraction of *A. baylyi* strain ADP1200-2 sonicated for 5 minutes.

Gel fragment	ng/µl	Total yield in µg	260/280
up to 1500 bp	43,4	1,30	1,90
1500 to 3000 bp	10,3	0,31	1,81
3000 to 10000 bp	7,9	0,24	1,56

It was then tried to use a larger volume (20 µl) of DNA. *A.baylyi* strain ADP1200-2 was sonicated for 3 minutes, the fragment size is shown in figure 15.

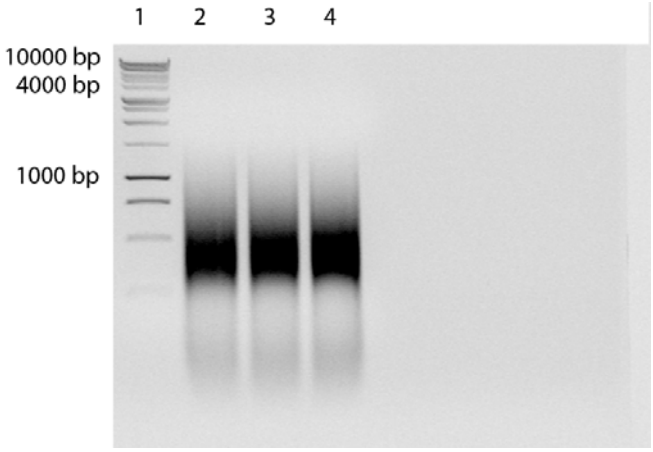

Figure 15. Isolated DNA of *A. baylyi* strain ADP1200-2 sonicated for 180 seconds (3 minutes). Lanes: 1: 1 Kb DNA ladder (AB gene, UK), 2 to 4: isolated DNA of ADP1200-2 sonicated for 180 seconds (image is inverted).

Three gel pieces were cut out in the size range 250 to 1000 bp, 1000 to 3000 bp and 3000 to 10000 bp. The extracted DNA from the gel pieces was used as donor DNA in filter transformation. Even though a larger volume of DNA was applied to the wells, the yield of extracted DNA was just a little bit higher and there were no resulting transformants. The yield of DNA is shown in table 10.

Table 10. Yield from DNA extraction of *A. baylyi* strain ADP1200-2 sonicated for 180 seconds.

Gel fragment	ng/µl	Total yield in µg	260/280
250 to 1000 bp	222,4	6,67	1,90
1000 to 3000 bp	14,1	0,42	1,99
3000 to 10000 bp	12,9	0,39	1,94

The low yield of extracted DNA and the use of EtBr in the gel were thought to be the reason for this since EtBr is toxic and would probably reduce the TF. An agarose gel was therefore made without EtBr. 5 µl EtBr was mixed with the ladder before it was applied to the first well and 40 µl DNA was applied to five wells. This time the whole gel fraction was centrifuged through one column from the gel purifying kit to increase the yield of extracted DNA (earlier the gel fraction was divided into smaller pieces and these pieces were centrifuged through one column each). Three gel fractions were cut out in the size range up to 1000 bp, 1000 to 4000 bp and 4000 to 10000 bp. Two gel fractions were of the whole lane, one fraction was up to 10000 bp while the other also included the well and might include residues of unfragmented DNA. The yield of extracted DNA was a bit higher now and the filter transformation gave several transformants. The yield of the extracted DNA and the number of resulting transformants are shown in table 11.

Table 11. Yield from DNA extraction of *A. baylyi* strain ADP1200-2 sonicated for 180 seconds and the transformants from filter transformation in *A. baylyi* strain BD413. 100, 500 and 1300 µl of the undiluted bacterial DNA was plated on LBRK$^{50/50}$ plates and 100 µl on a LBR^{50} plate. The number of transformants on each plate is listed under the respective volumes of amount of bacterial DNA plated.

				Amount bacterial DNA plated		
Gel fragment	ng/µl	Total yield (µg)	260/280	100 µl	500 µl	1300 µl
up to 1000 bp	494.7	14.84	1.87	0	0	1
1000 to 4000 bp	12.2	0.37	1.69	9	22	~300
4000 to 10000 bp	29.7	0.89	1.55	0	4	1
the whole lane[a]	134.8	4.04	1.85	3	19	51
the whole lane[b]	127.8	3.83	1.88	15	90	TNTC

[a] the whole lane up to 10000 bp, [b] the whole lane including the well, TNTC= too numerous to count, 0.9% NaCl was used as negative control, there was no growth on the LBRK$^{50/50}$ plates with 0.9% NaCl and competent cells because *A. baylyi* strain BD413 is not resistant against kanamycin, unsonicated *A. baylyi* strain ADP1200-2 was used as positive control and there was lawn growth on these LBRK$^{50/50}$ plates. Because the filters from the filter transformation were washed with 0.9% NaCl and undiluted bacterial DNA was plated, the total number of transformants is the number listed in the table multiplied by 20.

3.2.4 Agarose gel staining with EtBr and SYBR

When DNA was extracted from gel fractions with EtBr there were no resulting transformants, but when DNA was extracted from a gel fraction without EtBr there was resulting transformants (table 11). It was much easier to cut out gel fractions from a stained gel. To avoid any toxic effect from EtBr, the gel could be stained in SYBR or the EtBR gel could be destained after the electrophoresis. To test this, one 1% agarose gel was stained with EtBr and one gel was stained with SYBR (which is not toxic) (see material and methods for staining of

gel with EtBr and SYBR). 40 µl of DNA from 16.4.0 total population sonicated for 10 seconds was divided into two wells in the gel with EtBr and 40 µl was divided into two of the wells in the gel that would be stained in SYBR. Gel fractions between 1000 and 4000 bp were cut out from the gels (one fraction contained two lanes in the gel). DNA was extracted from the gel fractions and used in filter transformation. Table 12 shows the yield of the extracted DNA and the number of the resulting transformants and figure 16 shows the fragment size of DNA from 16.4.0 extracted from one agarose stained with EtBr and one agarose gel stained with SYBR.

Table 12. Yield from extracted DNA of 16.4.0 total population sonicated for 10 seconds and the resulting transformants when the DNA was used as donor in filter transformation in *A. baylyi* strain BD413. 100, 500 and 1300 µl of the undiluted bacterial DNA was plated on LBRK$^{50/50}$ plates and 100 µl on a LBR^{50} plate. The number of transformants on each plate is listed under the respective volumes of amount of bacterial DNA plated.

				Amount bacterial DNA plated		
Gel fragment[a]	ng/µl	Total yield in µg	260/280	100 µl	500 µl	1300 µl
gel with EtBr	199.6	5.9	1.84	TNTC	TNTC	TNTC
gel stained in SYBR	231.7	6.95	1.85	266	TNTC	TNTC

[a] the size of the gel fragments were 1000 to 4000 bp, TNTC= too numerous to count, 0.9% NaCl was used as negative control, there was no growth on the LBRK plates with 0.9% NaCl and competent cells because *A.. baylyi* strain BD413 is not resistant against kanamycin, unsonicated *A. baylyi* strain ADP1200-2 was used as positive control, there was lawn growth on the LBRK plates used. Because the filters from the filter transformation were washed with 0.9% NaCl and undiluted bacterial DNA was plated, the total number of transformants is the number listed in the table multiplied by 20.

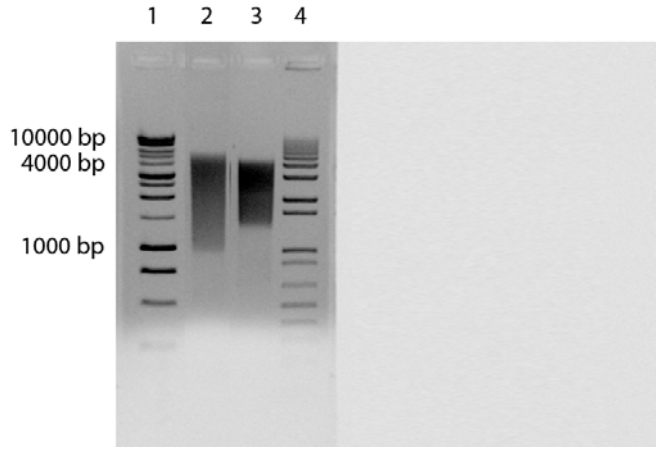

Figure 16. Isolated DNA of 16.4.0 total population sonicated for 10 seconds extracted from agarose gel. Lanes: 1 and 4: 1 Kb DNA ladder (AB gene, UK), 2: DNA from 16.4.0 total population sonicated for 10 seconds from agarose gel with EtBr, 3: DNA from 16.4.0 total population sonicated for 10 seconds from agarose gel stained with SYBR (image is inverted).

There was good growth of transformants both on the plates with DNA extracted from the gel with EtBr and from the gel stained in SYBR (table 12). To test if EtBr affects the transformation frequency, 1 µl EtBr was added to 65 µl of *A. baylyi* ADP1200-2 sonicated for 3 minutes before it was mixed with competent cells of *A. baylyi* strain BD413. The transformation frequency was compared to the transformation frequency of *A. baylyi* ADP1200-2 sonicated for 180 seconds (3 minutes) without EtBr. There was no difference in the transformation frequencies as shown in table 13.

Table 13. Transformationfrequency of unsonicated *A. baylyi* strain ADP1200-2 and sonicated *A. baylyi* strain ADP1200-2 for 180 seconds with and without EtBr.

Strain ADP1200-2	Average transformation frequency	Standard deviation
Unsonicated	1,9E-03	2,8E-04
180 s sonicated	4,8E-06	1,0E-06
180 s sonicated with 1 µl EtBr	4,8E-06	1,1E-06

3.3 Introgression

Because EtBr did not seem to affect the TF (table 13), the agarose gels in the introgression study from generation 1 and to generation 6 were made with EtBr. EtBr was removed by washing the gel pieces with dH_2O before DNA was extracted from them. Gel fractions between 1000 and 4000 bp were cut out from the gel (except for in the initial filter transformation) and extracted DNA from these were used as donor DNA in the filter transformations.

3.3.1 Backcross generations

Six backcross transformations were performed with *A. baylyi* strain BD413 as recipient after the initial transformation with DNA from *A.* sp. strain 16.4 as donor. Transformants resulting from the initial transformation were called 16.4.0 and this was generation 0. Transformants resulting from the next transformation with isolated, sonicated DNA from 16.4.0 as donor, were called 16.4.1 and this was generation 1 and so on.

One backcross transformation was performed with *A. baylyi* ADP7021 as recipient after the initial transformation with DNA from *A.* sp. 16.4 as donor. Transformants resulting from the initial transformation were called MS16.4.0 (MS= mutator single colony) and this was

generation 0. Transformants resulting from the next transformation with DNA from MS16.4.0 as donor were called MS16.4.1 and this was generation 1.

Introgression in the wild type

After the initial transformation with isolated DNA from *A.* sp. 16.4 as donor DNA, DNA from the resulting transformants was used in subsequent backcross transformations with *A. baylyi* strain BD413 as recipient. Table 4 in the Appendix shows the number of the resulting transformants from each generation.

DNA from *A.* sp. strain 16.4 sonicated for 5 and 10 seconds was used as donor DNA in the initial filter transformation with *A. baylyi* strain BD413 as recipient. Genomic DNA was isolated from a total population of all the 19 resulting transformants (both from 5 and 10 seconds) as shown in table 4 in the Appendix. This DNA was called 16.4.0 total population and sonicated for 10 seconds. Sonicated DNA from 16.4.0 total population was applied to an agarose gel with EtBr and one agarose gel that was stained in SYBR. Figure 17 shows the fragment size of DNA from 16.4.0 total population sonicated for 10 seconds on an agarose gel.

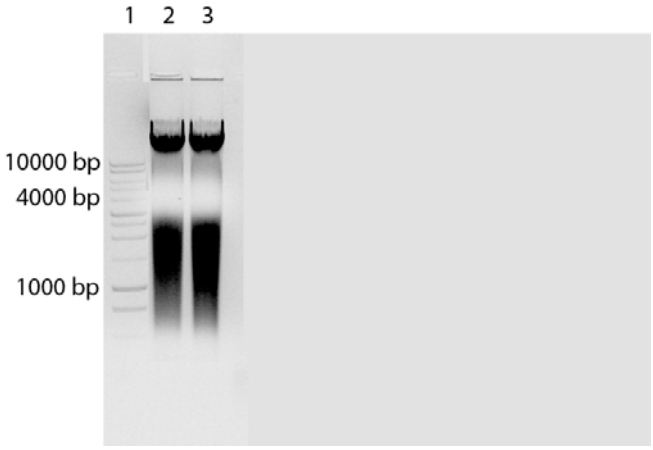

Figure 17. Isolated DNA of 16.4.0 total population sonicated for 10 seconds. Lanes: 1: 1 Kb DNA ladder (AB gene, UK), 2 and 3: DNA from 16.4.0 sonicated for 10s (image is inverted).

A fraction between 1000 to 4000 bp was cut out (one piece with both lanes) from the gel stained in SYBR with DNA from 16.4.0 sonicated for 10 seconds (picture not available). The yield from the DNA extraction was 6.95 µg (table 12).

The extracted DNA was used as donor in filter transformation with *A. baylyi* strain BD413. This resulted in 266 transformants called 16.4.1 total population. Genomic DNA was isolated from 16.4.1 total population and fragmented by sonication for 10 seconds to 3 minutes. The size of the fragmented DNA is shown in figure 18.

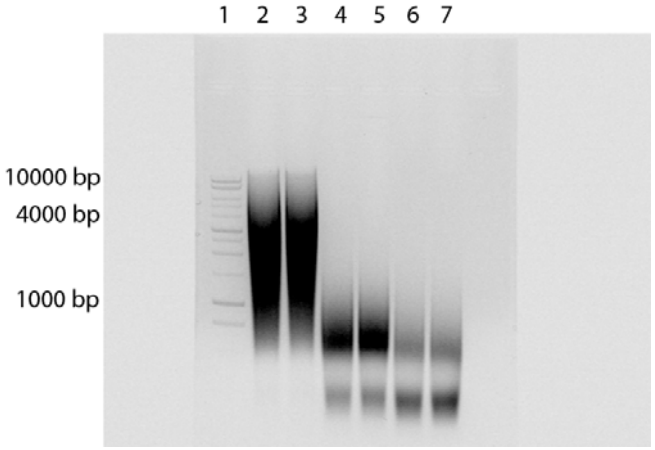

Figure 18. Isolated DNA of 16.4.1 total population sonicated for 10 seconds to 3 minutes. Lanes: 1: 1 Kb DNA ladder (AB gene, UK), 2 and 3: DNA from 16.4.1 tot.pop. sonicated for 10s, 4 and 5: DNA from 16.4.1 total population sonicated for 60s, 6 and 7: DNA from 16.4.1 total population sonicated for 3min (image is inverted).

A fraction between 1000 to 4000 bp was extracted from the lanes in the gel with 16.4.1 sonicated for 60 seconds. The yield from the DNA extraction was 1.3 µg (table 12). The size of the extracted DNA was from around 1250 to 4000 bp. Figure 19 shows the fragment size of sonicated DNA from 16.4.1 extracted from an agarose gel.

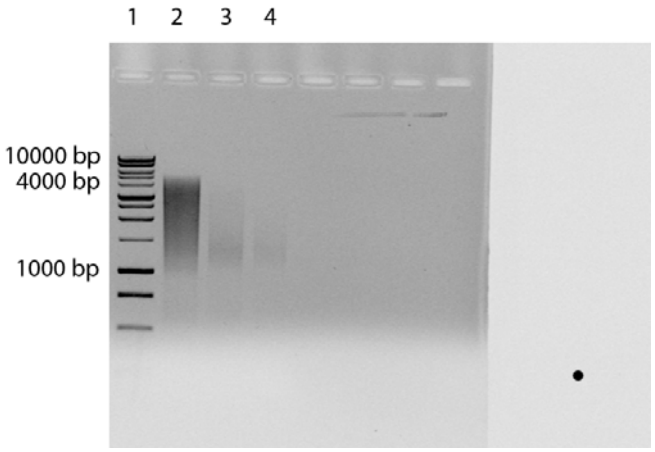

Figure 19. Isolated DNA of 16.4.1 total population sonicated for 10s to 3 min extracted from agarose gel. Lanes: 1: 1 Kb DNA ladder (AB gene, UK), 2: DNA from 16.4.1 total population sonicated for 10s, 3: DNA from 16.4.1 total population sonicated for 60s, 4: DNA from 16.4.1 total population sonicated for 180s (image is inverted).

The extracted DNA was used as donor in filter transformation with *A. baylyi* strain BD413. This resulted in 62 transformants, when the donor DNA was sonicated for 1 minute (see table 4 in the Appendix). Genomic DNA was isolated from the resulting transformants, called 16.4.2 total population, and sonicated for 1 minute. Figure 20 shows the fragment size of sonicated DNA from 16.4.2 total population.

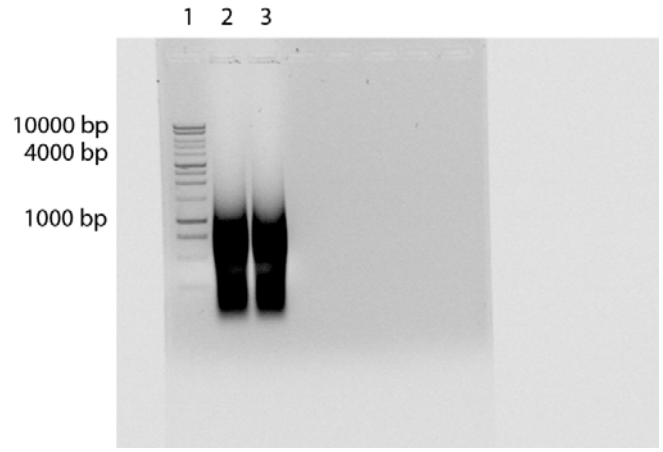

Figure 20. Isolated DNA of 16.4.2 total population sonicated for 1 minute. Lanes: 1: 1 Kb DNA ladder (AB gene, UK), 2 and 3: DNA from 16.4.2 total population sonicated for 60s (image is inverted).

A fraction between 1000 to 4000 bp was extracted from the two lanes in the gel. The yield from the DNA extraction was 1.0 µg (table 12). The size of the extracted DNA used as donor DNA was from 1000 to 4000 bp. Figure 21 shows the fragment size of sonicated DNA from 16.4.2 total population extracted from an agarose gel.

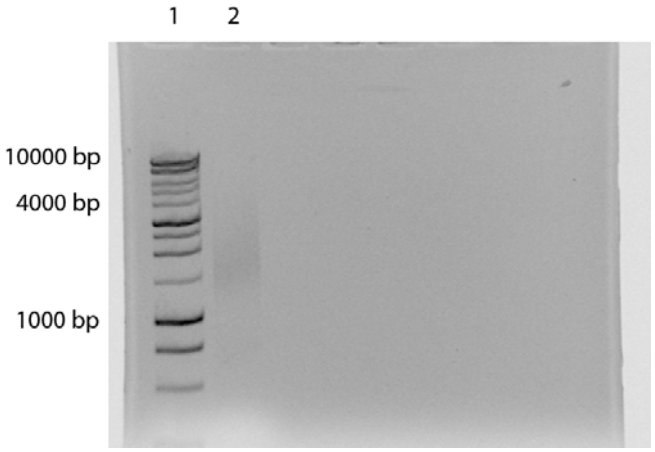

Figure 21. Isolated DNA of 16.4.2 total population sonicated for 1 minute extracted from agarosgel. .Lanes: 1: 1 Kb DNA ladder (AB gene, UK), 2: DNA from 16.4.2 total population sonicated for 60s (image is inverted).

The size of the extracted DNA used as donor DNA in generation 3 was from 1000 to 4000 bp. The extracted DNA was used as donor in filter transformation with *A. baylyi* strain BD413. Genomic DNA isolated from 11 of the resulting transformants (see table 4 in Appendix), was called 16.4.3 total population and fragmented by sonication for 1 minute (image of sonicated DNA from 16.4.3 total population and image of fragment size of extracted DNA from 16.4.3 is not available.).

After another round of filter transformation in *A. baylyi* strain BD413, genomic DNA was isolated from the 134 resulting transformants (see table 4 in Appendix). This DNA was called 16.4.4 total population and fragmented by sonication for 1 minute. The size of the sonicated DNA from 16.4.4 total population is shown in figure 22.

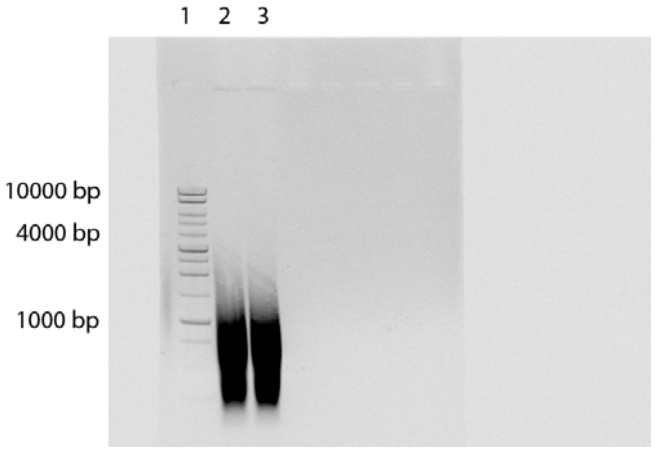

Figure 22. Isolated DNA of 16.4.4 total population sonicated for 1 minute. Lanes: 1: 1 Kb DNA ladder (AB gene, UK), 2 and 3: DNA from 16.4.4 total population sonicated for 60s (image is inverted).

DNA from 16.6.4 total population was extracted from the agarose gel (image not available) and used as donor DNA in filter transformation with *A. baylyi* strain BD413. There were no transformants after the filter transformation so DNA from 16.4.4 total population was sonicated for 10 and 30 seconds. Figure 23 shows the size of DNA from 16.4.4 total population sonicated for 10 and 30 seconds.

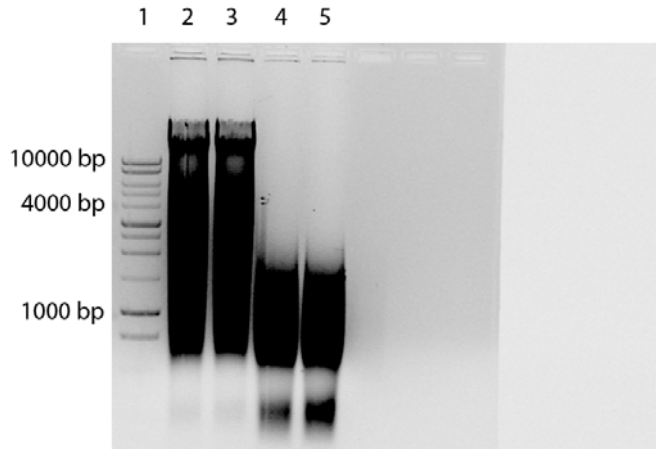

Figure 23. Isolated DNA of 16.4.4 total population sonicated for 10-30 seconds. Lanes: 1: 1 Kb DNA ladder (AB gene, UK), 2 and 3: DNA from 16.4.4 total population sonicated for 10s, 4 and 5: DNA from 16.4.4 total population sonicated for 30s (image is inverted).

A fraction between 1000 to 4000 bp was extracted from the lanes in the gel. The yield from the DNA extraction when the DNA was sonicated for 10 seconds was 1.4 µg (table 12). Figure 24 shows the fragment size of DNA from 16.4.4 total population sonicated for 10 and 30 seconds and extracted from an agarose gel.

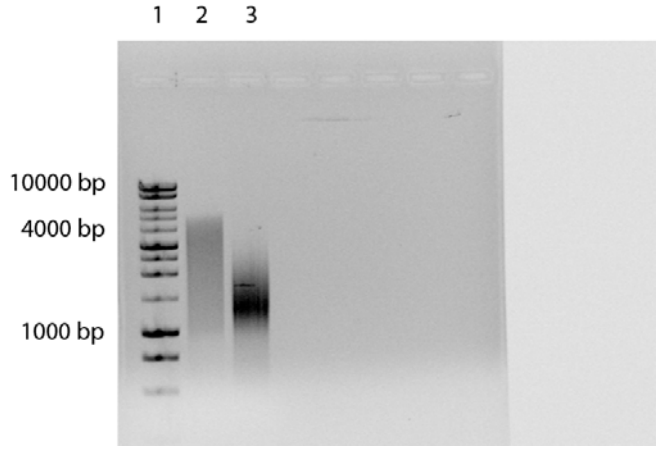

Figure 24. Isolated DNA of 16.4.4 total population sonicated for 10 to 30 seconds extracted from agarosegel. Lanes: 1: 1 Kb DNA ladder (AB gene, UK), 2: DNA from 16.4.4 total population sonicated for 10s, 3: DNA from 16.4.4 total population sonicated for 30s (image is inverted).

The size of the extracted DNA was from 1000 to 4000 bp (after 10 seconds of sonication) and it was used in filter transformation with *A. baylyi* strain BD413. Genomic DNA was isolated from the 172 resulting transformants (see table 4 in Appendix). This DNA was called 16.4.5 total population and fragmented by sonication for 10 seconds. The size of sonicated DNA from 16.4.5 total population is shown in figure 25.

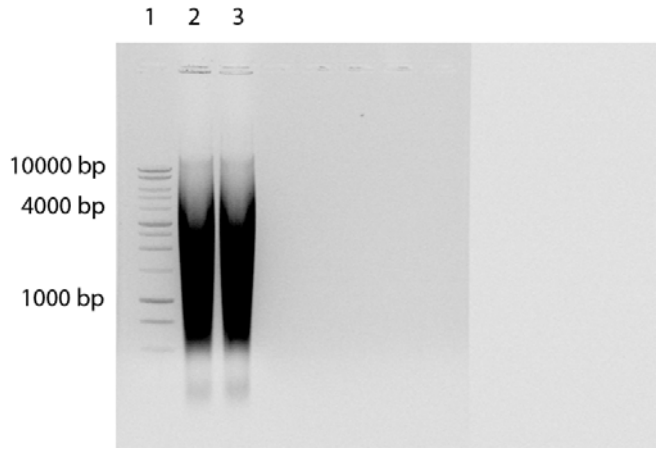

Figure 25. Isolated DNA of 16.4.5 total population sonicated for 10 seconds. Lanes: 1: 1 Kb DNA ladder (AB gene, UK), 2 and 3: DNA from 16.4.5 total population sonicated for 10s (image is inverted).

A fraction between 1000 to 4000 bp was extracted from the lanes in the gel. The yield from the DNA extraction was 5.8 µg (table 12). Figure 26 shows the fragment size of sonicated DNA from 16.4.5 total population extracted from an agarose gel.

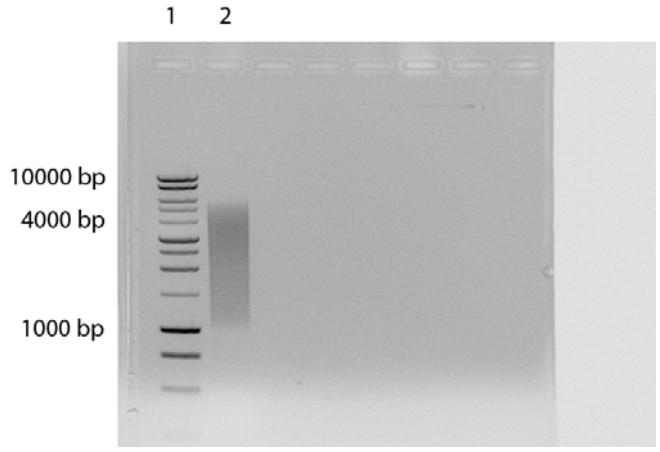

Figure 26. Isolated DNA of 16.4.5 total population sonicated for 10 seconds extracted from agarosegel. Lanes: 1: 1 Kb DNA ladder (AB gene, UK), 2: DNA from 16.4.5 total population sonicated for 10s (image is inverted).

The size of the extracted DNA was from 1000 to almost 5000 bp. Genomic DNA was isolated from the 347 resulting transformants (see table 4 in Appendix), called 16.4.6 total population. This was the last step in the introgression study of the wild type.

Introgression in the mutator strain.

After the initial transformation with *A.* sp. strain 16.4 as donor DNA and *A. baylyi* strain ADP7021 as recipient, DNA from the resulting transformants was used in subsequent backcross transformations with *A. baylyi* strain ADP7021 as recipient. Table 5 in Appendix shows the number of the resulting transformants from each generation.

Isolated DNA from *A.* sp. strain 16.4 was sonicated for 5 and 10 seconds and used as donor DNA in the initial filter transformation with *A. baylyi* strain ADP7021 as recipient. Only donor DNA sonicated for 5 seconds gave resulting transformants (see table 5 in Appendix). Genomic DNA was isolated from 5 single colonies, called MS16.4.0.1-5 (Mutator Single colonies, 16.4.0 is the transformants from the initial transformation and 1-5 means single colony 1 to single colony 5), and fragmented by sonication for 10 seconds. Figure 27 shows the fragment size of MS16.4.0.1-5 sonicated for 10 seconds.

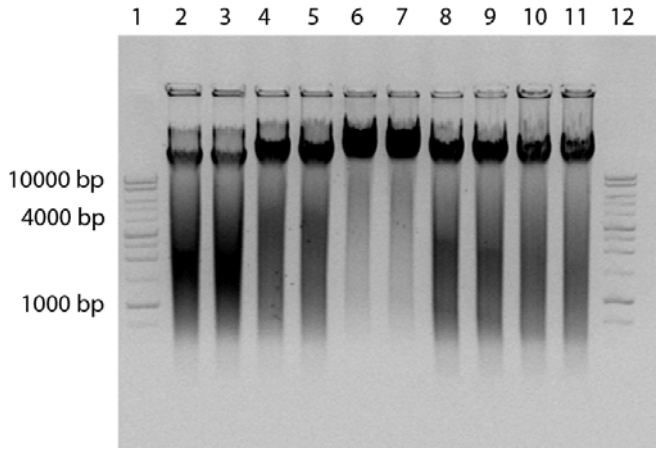

Figure 27. Isolated DNA of MS16.4.0.1-5 sonicated for 10 seconds. Lanes: 1 and 12: 1 Kb DNA ladder (AB gene, UK), 2 and 3: DNA from MS16.4.0.1, 4 and 5: DNA from MS16.4.0.2, 6 and 7: DNA from MS16.4.0.3, 8 and 9: DNA from MS16.4.0.4, 10 and 11: DNA from MS16.4.0.5 (image is inverted)

DNA from MS16.4.0.1-5 was extracted from the agarose gel and used as donor DNA in filter transformations with *A. baylyi* strain ADP7021. There were no resulting transformants after the filter transformations (see table 5 in Appendix), so DNA from MS16.4.0.1-5 was sonicated for 5 seconds, extracted from the gel pieces and used as donor DNA in filter transformation with *A. baylyi* strain ADP7021 as recipient. The size of fragmented DNA from MS16.4.0.1-5 sonicates for 5 seconds is shown in figure 28.

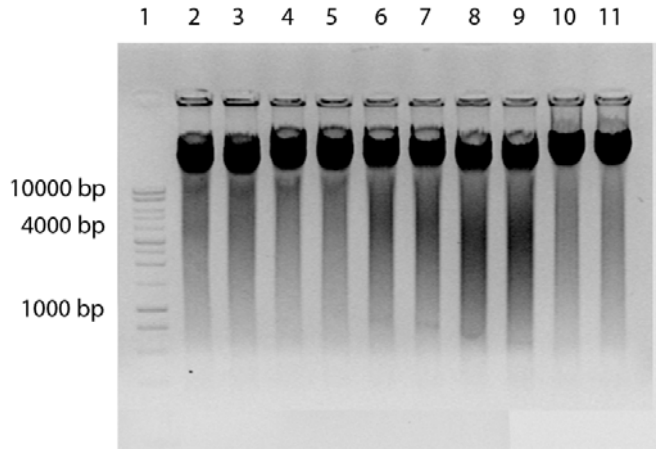

Figure 28. Isolated DNA of MS16.4.0.1-5 sonicated for 5 seconds. Lanes: 1: 1 Kb DNA ladder (AB gene, UK), 2 and 3: DNA from MS16.4.0.1, 4 and 5: DNA from MS16.4.0.2, 6 and 7: DNA from MS16.4.0.3, 8 and 9: DNA from MS16.4.0.4, 10 and 11: DNA from MS16.4.0.5 (image is inverted).

Genomic DNA was isolated from the resulting transformants (see table 5 in Appendix), called MS16.4.1.1-5. This was the last step in the introgression study of the mutator strain.

DNA extracted from the agarose gels in the introgression studies

As mentioned earlier in 3.3.1, DNA was extracted from the agarose gels with sonicated DNA from the transformants in generation 0 to generation 6 in the introgression study with the wild type and in generation 0 to generation 1 in the introgresson study with the mutator strain. The DNA was eluted from the column membrane by adding 30 µl dH_2O and the concentration was measured before the DNA was used in filter transformation. Table 12 shows the yield of DNA extracted from sonicated DNA of generation 0 and table 14 shows the yield of DNA extracted from sonicated DNA of generation 1 to generation 6 in the wild type. Table 15 shows the yield of DNA extracted from single colonies of sonicated DNA of generation 0 and generation 1 in the mutator strain.

Table 14.Yield from DNA extraction from agarose gel.

Sample	ng/µl	Total yield in µg	260/280
16.4.1, sonicated for 1 min	43.5	1.30	1.90
16.4.2, sonicated for 1 min	32.6	0.98	1.62
16.4.3, sonicated for 1 min	N/A	N/A	N/A
16.4.4, sonicated for 10 sec	45.0	1.35	1.82
16.4.5, sonicated for 10 sec	194.6	5.84	1.84

N/A= not available

Table 15. Yield from DNA-extraction from agarose gel.

Sample	ng/µl	Total yield in µg	260/280
MS16.4.0.1, sonicated for 5 sec	84.77	2.54	1.85
MS16.4.0.2, sonicated for 5 sec	24.94	0.75	1.72
MS16.4.0.3, sonicated for 5 sec	8.04	0.24	1.65
MS16.4.0.4, sonicated for 5 sec	72.41	2.17	1.85
MS16.4.0.5, sonicated for 5 sec	27.36	0.82	1.91

To check if the extracted DNA had the desirable fragment size, 2-5 µl were applied to an agarose gel (figures are shown in section 3.3.1).

3.3.2 Sequencing of flanking DNA

Genomic DNA was isolated from five single colonies in generation 0 and from five single colonies in generation 5 in the wild type line and sequenced with different primers (see primer map, figure 1 in the Appendix). Table 16 shows the left flanking regions from the *npt*II gene and table 17 shows the right flanking regions from the *npt*II gene.

Table 16. Sequenced left *npt*II flanking regions of transformants in *A. baylyi* strain BD413 (single colonies).

	Primer				
	RP152		RP255	Apr27	RV20
Isolate	BD413 hit	Overlap with nptII	BD413 hit	BD413 hit	BD413 hit
S16.4.0.1	No hit	130 bp	-	-	Hit
S16.4.0.2	No sequence	no	No hit	No sequence	Hit
S16.4.0.3	No sequence	no	No hit	Hit	No sequence
S16.4.0.4	No hit	125 bp	-	-	Hit
S16.4.0.5	No hit	no	No hit	No sequence	Hit
S16.4.5.1	No sequence	no	No hit	Hit	-
S16.4.5.2	No sequence	no	No hit	Hit	-
S16.4.5.3	No sequence	no	No hit	Hit	-
S16.4.5.4	No sequence	no	No hit	Hit	-
S16.4.5.5	No sequence	no	No hit	Hit	-

Hit indicates 100% query sequence aligned with ***A. baylyi*** strain BD413 in the same region, discrepancies was due to unresolved nucleotides in the query, blasted in average 700 nucleotides, there was only overlap with *npt*II when primer RP152 was used; -indicates that this primer was not used for this sequence.

Table 17. Sequenced right *npt*II flanking regions of transformants in *A. baylyi* strain BD413.

	Primer				
		LP1046	LP853	Apr25	FW24
Isolate	BD413 hit	Overlap with nptII	BD413 hit	BD413 hit	BD413 hit
S16.4.0.1	No hit	145 bp	-	-	No sequence
S16.4.0.2	Hit	no	No sequence	No sequence	No sequence
S16.4.0.3	Hit	no	No sequence	No sequence	No sequence
S16.4.0.4	No hit	145 bp	-	-	No sequence
S16.4.0.5	Hit	no	No sequence	No sequence	No sequence
S16.4.5.1	Hit	no	No sequence	No sequence	-
S16.4.5.2	Hit	no	No sequence	No sequence	-
S16.4.5.3	Hit	no	No sequence	No sequence	-
S16.4.5.4	Hit	no	No sequence	No sequence	-
S16.4.5.5	Hit	no	No sequence	No sequence	-

Hit indicates 100% query sequence aligned with ***A. baylyi*** strain BD413 in the same region, discrepancies was due to unresolved nucleotides in the query, blasted in average 700 nucleotides, there was only overlap with *npt*II when primer LP1046 was used; - indicates that this primer was not used for this sequence

Genomic DNA was isolated from five single colonies in generation 0 and from five single colonies in generation 1 in the mutator line and sequenced with one forward and one reverse primer. Table 18 shows the flanking regions from the *npt*II gene.

Table 18. Sequenced flanking regions of transformants in *A. baylyi* strain ADP7021

Left *npt*II flanking region			Right *npt*II flanking region	
Primer RP152			Primer LP1046	
BD413 hit	Overlap with nptII	Isolate	Overlap with nptII	BD413 hit
No hit	100 bp	MS16.4.0.1	145 bp	No hit
No hit	125 bp	MS16.4.0.2	145 bp	No hit
Hit*	no	MS16.4.0.3	145 bp	No hit
No hit	120 bp	MS16.4.0.4	150 bp	No hit
No hit	130 bp	MS16.4.0.5	145 bp	No hit
Hit	no	MS16.4.1.1	no	No sequence
Hit	no	MS16.4.1.2	no	No sequence
Hit	no	MS16.4.1.3	no	No sequence
Hit	no	MS16.4.1.4	no	No sequence
Hit	no	MS16.4.1.5	no	No sequence

Hit indicates 100% query sequence aligned with *A. baylyi* strain BD413 in the same region, discrepancies was due to unresolved nucleotides in the query, blasted in average 700 nucleotides

4. DISCUSSION

4.1 DNA yield and purity

Isolation of DNA gave an average yield with a concentration of 2.0 µg/µl. We only needed DNA with a concentration of 0.5 µg/µl for use in the filter transformations, so the yield was high. The concentration of the isolated DNA was measured with the NanoDrop® ND-1000 spectrophotometer. One of the parameters was the ratio 260/280, which is the ratio of sample absorbance at 260 and 280 nm. It is used to assess the purity of DNA. A ratio of ~1.8 is thought of as pure for DNA. If the ratio is lower it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. When the DNA concentration of isolated from *A. baylyi* ADP1200-2, *A.* sp. strain 16.4 and resulting transformants in *A. baylyi* strain BD413 or strain ADP7021 was measured, the ratio 260/280 was ~1.8. When the DNA concentration of the extracted DNA was measured, the ratio 260/280 was mostly ~1.8, but for strain 16.4.2 (total population) (table 14) and strain MS16.4.0.3 (table 15) it was lower (1.62 and 1.65 respectively). These were not as pure as the other extracted DNA, but they still gave resulting transformants in filter transformations with the wild type and the mutator strain. As we did not want to measure the transformation frequency (TF) in the introgression study, we could use these transformants in the next round of backcross transformations.

4.2 Method for optimal fragmentation of DNA

We tried two different methods for fragmentation of DNA, nebulization and sonication.

4.2.1 DNA fragmentation by nebulization

A. baylyi strain ADP1200-2 was fragmented by nebulization. Fragmentation of DNA by nebulization gave as expected a gradual reduction in DNA fragment size during increasing time of nebulization. Because the Shearing buffer was added to the DNA before the nebulization, it was necessary to precipitate the DNA to get pure DNA that could be used in filter transformation. Figure 7 shows a gradual reduction in fragments size for the nebulized DNA. The yield of precipitated DNA varied for nebulized DNA of *A. baylyi* strain ADP1200-

2 (table 5). The TF did not decrease gradually (table 6), as we expected from the size of the DNA fragments (fig. 7). It could be that the DNA pellet was not completely dissolved after the precipitation thus the DNA concentration could be inaccurate. Even though the top of the nebulizer was tightened, there was a great loss of liquid during nebulization. The loss of liquid during the nebulization made it necessary to use another method to fragmentate DNA.

4.2.2 DNA fragmentation by sonication

A. baylyi strain ADP1200-2 and *A.* sp. strain 16.4 were fragmented by sonication. As mentioned in the introduction, *A.* sp. strain 16.4 is the same as *A*. sp. 62A1. Fragmentation of DNA by sonication gave an effective, gradual reduction in DNA fragment size during increasing time of sonication (fig. 9). Because only DNA dissolved in dH_2O (with a concentration of 0.5 µg/µl) was sonicated (no added buffer), it was not necessary to precipitate the DNA before further use. Combined with the fact that there was no loss of liquid during sonication made it an effective and easy method to fragment DNA. For longer sonications (over 7 minutes) the water temperature increased and the water had to be replaced with new ice and cold (4ºC) water to avoid increasing temperature and to keep the conditions the same for all samples during sonication. Too much ice in the tank could block the sound waves or keep the tubes from reaching the water, which would reduce the effect of fragmentation. Also the number of samples sonicated could effect the fragmentation. Five tubes with samples might absorb more sound waves than one tube. The size of the sonicated DNA was checked on agarose gels (fig. 9 and 10), to control the degree of fragmentation. There was a gradual reduction in TF with increasing length of sonication for DNA , except for DNA of *A. baylyi* strain ADP1200-2 sonicated for 3600 seconds (60 minutes) (table 7), were the TF was similar to the TF after sonication for 180 seconds. Figure 10 shows that the fragmentation for 60 minutes for DNA from *A. baylyi* strain ADP1200-2 was incomplete. The ice was changed several times during this sonication and could explain the poor sonication. Many studies using *A. baylyi* strain BD413 have shown that sequence homology of regions flanking the DNA to be transferred affect the TF (Simpson et al., 2007). When fragmented donor DNA from *A. baylyi* strain ADP1200-2 was used in filter transformation with *A. baylyi* strain BD413, the TF decreased whit increasing fragmentation. The decrease in TF is explained by the decreasing size of sequence homology.

The TF of *A.* sp. strain 16.4 was almost the same when the DNA was unsonicated or sonicated for 5 or 10 seconds (table 8). Figure 12 and 13 show that there is a significant amount of unfragmentd DNA left. This is probably the DNA that contributes most to the TF because after 10 seconds, when unfragmented DNA is eliminated, the TF decreases below detection. The detection limit of the assay was approximately $1*10^{-10}$ transformants per recipient. *A.* sp. strain 16.4 is 24.4% sequence divergent from BD413 and this could explain the difficulty of the initial transformation of *A.* sp. strain 16.4 DNA in *A. baylyi* strain BD413. *A.* sp. strain 16.4 DNA sonicated for longer than 10 seconds gave no transformants. A longer donor fragment may lend more stability to the heteroduplex intermediate (Zawadzki et al., 1995), while a shorter donor fragment (e.g. by sonication) may not form such a stable heteroduplex intermediate. Also the stability of the recipient-donor heteroduplex is dependent on the degree of sequence similarity between donor and recipient DNA (Zawadzki et al., 1995). Longer fragmentation of divergent DNA has a lower probability of finding homologous regions for integration.

Because nebulization had some technical limitations, the DNA used as donor DNA in the introgression study was fragmented only by sonication.

4.2.3 Extraction of DNA from agarose gel

When we extracted sonicated DNA from a specific part of the agarose gel, we were sure that only fragmented DNA was used as donor DNA in the filter transformation. It took quite some time to find the right method to do this and the challenges were to find the right volume of DNA applied to the well and the right fragment size and to get maximum yield of DNA extracted from the gel so that it could be used as donor DNA in filter transformations. When 10 µl DNA was applied to each well and DNA was extracted from different fractions of the gel, there were no resulting transformants, so we tried to apply 20 µl DNA in each well. Even though we doubled the amount of DNA, there were no resulting transformants. A small amount of DNA and the size of the gel fragments extracted (see table 9 and 10) could explain this. The fragment size could be too low in fractions from, e.g. 250 to 1000 bp (this fraction did not include the *npt*II gene) to give transformants when used as donor DNA in filter transformations. When high fractions, e.g. 3000 to 10000 bp, were cut out from the gel, there were probably too few fragments in this fraction to give transformants when used as donor DNA in filter transformations. Figure 15 shows that most of the fragments have a size around

500 bp, thus there would only be few fragments in the high fraction (3000 to 10 000 bp). Also EtBr or exposure to ultraviolet (UV) radiation could affect the DNA. When 40 µl DNA was applied to each well on an unstained gel (5 µl EtBr was added to the ladder), there were several transformats (table 11). The fractions that gave the highest number of transformants were the fraction from 1000 to 4000 bp and the fraction of the whole lane including the well. The lower limit (1000 bp) was chosen because the size of the *npt*II is around 1220 bp and we wanted to be sure that the fraction included the gene. The upper limit (4000 bp) was chosen because we wanted some flanking regions in addition to the *npt*II gene, but we also wanted to limit the length of flanking regions. Simpson et al. (2007) showed that a minimum of 500 bp was required on each flank for transformation to be affected by flanking homology. The fraction of the whole lane including the well probably contained some unfragmented DNA, which would make the integration easier and explain the high number of transformants from this fraction.

4.2.4 Agarose gel staining with EtBr and SYBR

It was easier to cut out gel fractions from a stained gel than an unstained gel because we could visually inspect both the gel run and fragmentation. We tried to stain the gel in SYBR, because it was hypothesized that EtBr could affect the TF because it interchelates with DNA and thereby having a toxic effect on the cells. Staining of the gel with SYBR or staining with EtBr and then distaining the gel before DNA was extracted from the gel fractions, were both good methods for gel staining and both gave resulting transformants (table 12).

To test the effect of EtBr we compared to the TF of DNA from ADP1200-2 sonicated for 3 minutes with and without 1 µl EtBr added to the DNA. Table 13 shows that the transformation frequencies were the same ($4.8*10^{-6}$) and that EtBr did not seem to affect the TF. The gels in the introgression study were stained with EtBr.

4.3 Introgression

We were able to perform six backcross transformations in *A. baylyi* BD413 and one backcross transformation in *A. baylyi* ADP7021.

4.3.1 Backcross generations

We wanted to compare the TF when fragmented DNA of *A.* sp. strain 16.4 was used as donor DNA to the TF when unfragmentated DNA of *A.* sp. strain 16.4. In this study the TF for unsonicated and sonicated DNA of *A.* sp. strain 16.4 were equal (table 8), but the TF for unsonicated DNA of *A.* sp. strain 16.4 ($2.0*10^{-9}$) was lower than the same TF ($1.2*10^{-7}$) from a previous, unpublished introgression study (Ray et al.). The reason was probably contamination of *A.* sp. strain 16.4 (see below).

4.3.2 Sequencing of flanking DNA

A previous performed, unpublished introgression study (Ray et al.) presented evidence that unselected heterologous DNA might be eliminated from hybrid recombinant strains over many generations during introgression in a competent bacterial population. We wanted to determine how fragmentation of the donor DNA would affect the speed at which, and if unselected heterologous DNA was eliminated during introgression and compare these results to the results from the unpublished study (Ray et al.). To sequence identify cross-over junctions the resulting transformants from generation 0 and 5 in the wild type strain and generation 0 and 1 in the wild type strain, we used forward and reverse primers (see figure 1 in Appendix) that would bind to the *npt*II gene and to different sites at the genome (up to 6000 bp from the *npt*II gene). The *npt*II gene was only identified in S16.4.0.1 and S16.4.0.4 in generation 0, but in none of transformants in generation 5 in the wild type line. The *npt*II gene was identified in all the transformants in generation 0 in the mutator strain, but in none of the transformants in generation 1. This was unexpected, because we expected at least to identify the nptII gene in all the sequenced generations. When the sequences were used in a blastn analysis they either aligned 100% to *A. baylyi* BD413 or did not align at all (table 16 to 18). The sequencing results were not as expected, because the sequences from generation 0 in the wild type line in this study were not similar to the same sequences in the previous performed introgression study (Ray et al.). We isolated the donor DNA we used, *A.* sp. strain 16.4, again from the freeze stock and sequenced it, but it turned out that it was not the same DNA that was used in the previous performed introgression study (Ray et al.). It contained the *npt*II gene, but the sequences flanking right and left from the *npt*II were not as expected compared to the DNA used in the unpublished study (Ray et al.). We used blastn analysis to sequence the flanking regions of the *npt*II gene in the *A.* sp. strain 16.4 we had used. We found out that

the flanking regions were similar to an outer membrane protein in *A. baumannii*, thus our donor DNA isolated from the freeze stock and used in the introgression study was contaminated. This explained why the TF for unsonicated DNA of *A.* sp. strain 16.4 and the sequencing results were not as expected.

5. CONCLUSION

Introgression is the successive transformation of foreign DNA into the wild type population and is largely unknown in bacteria. One previous unpublished study investigated the effect of introgression of foreign unselected DNA, but the donor DNA used was of high molecular weight (around 20 to 30 kilo bases (kb)). I have developed a method for fragmentation of DNA to desired sizes and used this method to study introgression of unselected foreign DNA of 1000 to 4000 base pairs (bp). We wanted to determine how fragmentation of donor DNA will affect the speed at which, and if heterologous flanking DNA is eliminated during successive rounds of back transformation. The best fragmentation method was by sonication, which gives an effective, gradual reduction in DNA fragment size during increasing time of sonication. The size of the sonicated DNA is checked on an agarose gel and the appropriate gel fraction (1000 to 4000 bp in our setup) is cut out from the gel and used in filter transformation.

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Table 1. DNA isolated from ADP 1200-2, fragmentated by nebulization for 0 to 600 seconds. Number of colonies (CFU) on each plate. 3 parallels of each test on LBR plates and 3 parallels on LBRK plates. Calculated transformation frequency.

		LBR			LBRK									
Nebulizing	Test	1	2	3	1	2	3	Average CFU	Dilution	Average CFU transformants	Dilution	Total number recipients	Total number transformants	Transformation-frequency
	1	82	90	85	0	0	0	85,7	10^-7	0,0	10^0			
	2	55	67	66	0	0	0	62,7	10^-7	0,0	10^0			
NaCl	3	62	57	71	0	0	0	63,3	10^-7	0,0	10^0			
	4	49	88	87	172	189	194	74,7	10^-7	185,0	10^-4	3,0E+10	7,4E+07	2,5E-03
	5	99	88	103	267	271	269	96,7	10^-7	269,0	10^-4	3,9E+10	1,1E+08	2,8E-03
0 s	6	44	42	29	124	144	162	38,3	10^-7	143,3	10^-4	1,5E+10	5,7E+07	3,7E-03
	7	64	63	63	123	103	106	63,3	10^-7	110,7	10^-2	2,5E+10	4,4E+05	1,7E-05
	8	100	99	87	182	148	164	95,3	10^-7	164,7	10^-2	3,8E+10	6,6E+05	1,7E-05
40 s	9	73	90	83	146	149	171	82,0	10^-7	155,3	10^-2	3,3E+10	6,2E+05	1,9E-05
	10	84	93	124	49	45	66	100,3	10^-7	53,3	10^-1	4,0E+10	2,1E+04	5,3E-07
	11	75	83	77	60	76	57	78,3	10^-7	64,3	10^-1	3,1E+10	2,6E+04	8,2E-07
120 s	12	66	87	53	47	49	61	68,7	10^-7	52,3	10^-1	2,7E+10	2,1E+04	7,6E-07
	13	15	15	9	33	28	29	13,0	10^-7	30,0	10^-2	5,2E+09	1,2E+05	2,3E-05
	14	16	7	16	49	35	29	13,0	10^-7	37,7	10^-2	5,2E+09	1,5E+05	2,9E-05
240 s	15	17	31	24	34	41	45	24,0	10^-7	40,0	10^-2	9,6E+09	1,6E+05	1,7E-05
	16	89	120	97	76	84	92	102,0	10^-7	84,0	10^-1	4,1E+10	3,4E+04	8,2E-07
	17	61	77	57	67	44	53	65,0	10^-7	54,7	10^-1	2,6E+10	2,2E+04	8,4E-07
360 s	18	38	60	42	48	39	53	46,7	10^-7	46,7	10^-1	1,9E+10	1,9E+04	1,0E-06
	19	10	29	38	63	70	71	25,7	10^-7	68,0	10^-2	1,0E+10	2,7E+05	2,6E-05
	20	26	26	34	51	65	50	28,7	10^-7	55,3	10^-2	1,1E+10	2,2E+05	1,9E-05
600 s	21	45	21	26	62	54	75	30,7	10^-7	63,7	10^-2	1,2E+10	2,5E+05	2,1E-05

Table 2. DNA isolated from ADP 1200-2, fragmentated by sonication in 0 to 3600 seconds. Number of colonies (CFU) on each plate. 3 parallels of each test on LBR plates and 3 parallels on LBRK plates. Calculated transformation frequency.

		LBR			LBRK									
Sonication	Test	1	2	3	1	2	3	Average CFU recipients	Dilution	Average CFU transformants	Dilution	Total number recipients	Total number transformants	Transformation-frequency
	1	75	78	75	0	0	0	76,0	10^-7	0,0	10^0			
	2	90	109	95	0	0	0	98,0	10^-7	0,0	10^0			
NaCl	3	77	84	72	0	0	0	77,7	10^-7	0,0	10^0			
	4	97	97	97	27	37	34	97,0	10^-7	32,7	10^-5	3,9E+10	130666667	3,4E-03
	5	89	81	89	39	30	30	86,3	10^-7	33,0	10^-5	3,5E+10	132000000	3,8E-03
0 s	6	72	110	89	48	34	41	90,3	10^-7	41,0	10^-5	3,6E+10	164000000	4,5E-03
	7	89	90	74	68	62	70	84,3	10^-7	66,7	10^-2	3,4E+10	266667	7,9E-06
	8	109	112	110	116	82	112	110,3	10^-7	103,3	10^-2	4,4E+10	413333	9,4E-06
10 s	9	86	111	121	61	48	40	106,0	10^-7	49,7	10^-2	4,2E+10	198667	4,7E-06
	10	93	101	93	48	61	54	95,7	10^-7	54,3	10^-2	3,8E+10	217333	5,7E-06
	11	102	98	97	243	268	247	99,0	10^-7	252,7	10^-1	4,0E+10	101067	2,6E-06
60 s	12	98	75	86	152	212	202	86,3	10^-7	188,7	10^-1	3,5E+10	75467	2,2E-06
	13	112	113	116	238	301	259	113,7	10^-7	266,0	10^-1	4,5E+10	106400	2,3E-06
	14	98	118	120	183	264	273	112,0	10^-7	240,0	10^-1	4,5E+10	96000	2,1E-06
120 s	15	69	74	57	150	194	182	66,7	10^-7	175,3	10^-1	2,7E+10	70133	2,6E-06
	16	102	112	-	282	281	166	107,0	10^-7	281,5	10^-1	4,3E+10	112600	2,6E-06
	17	62	90	75	33	38	28	75,7	10^-7	33,0	10^-2	3,0E+10	132000	4,4E-06
180 s	18	85	79	76	299	299	294	80,0	10^-7	297,3	10^-1	3,2E+10	118933	3,7E-06
	19	88	85	99	177	202	188	90,7	10^-7	189,0	10^-1	3,6E+10	75600	2,1E-06
	20	112	85	69	235	186	170	88,7	10^-7	197,0	10^-1	3,5E+10	78800	2,2E-06
600 s	21	51	63	54	150	192	172	56,0	10^-7	171,3	10^-1	2,2E+10	68533	3,1E-06
	22	242	107	136	6	20	19	161,7	10^-7	15,0	10^-1	6,5E+10	6000	9,3E-08
	23	161	130	127	16	16	11	139,3	10^-7	14,3	10^-1	5,6E+10	5733	1,0E-07
1200 s	24	153	148	152	19	23	29	151,0	10^-7	23,7	10^-1	6,0E+10	9467	1,6E-07
	25	109	117	91	11	13	8	105,7	10^-7	10,7	10^-1	4,2E+10	4267	1,0E-07
	26	78	78	77	8	18	8	77,7	10^-7	11,3	10^-1	3,1E+10	4533	1,5E-07
1800 s	27	107	83	118	13	19	14	102,7	10^-7	15,3	10^-1	4,1E+10	6133	1,5E-07
	28	68	72	77	28	22	23	72,3	10^-7	24,3	10^-2	2,9E+10	97333	3,4E-06
	29	93	82	80	25	27	29	85,0	10^-7	27,0	10^-2	3,4E+10	108000	3,2E-06
3600 s	30	86	79	89	44	29	44	84,7	10^-7	39,0	10^-2	3,4E+10	156000	4,6E-06

Table 3. DNA isolated from 16.4, fragmentated by sonication in 0 to 10 seconds. Number of colonies (CFU) on each plate. 3 parallels of each test on LBR plates and 3 parallels on LBRK plates. Calculated transformation frequency.

		LBR			LBRK									
Sonication	Test	1	2	3	1	2	3	Average CFU recipients	Dilution	Average CFU transformants	Dilution	Total number recipients	Total number transformants	Transformation-frequency
	1	81	71	56	0	0	0	69,3	10^-7	0,0	10^0			
	2	63	81	83	0	0	0	75,7	10^-7	0,0	10^0			
NaCl	3	114	127	95	0	0	0	112,0	10^-7	0,0	10^0			
	4	133	82	128	78	72	66	130,5	10^-7	72,0	10^-5	5,2E+10	288000000	5,5E-03
ADP	5	126	120	119	68	66	59	121,7	10^-7	64,3	10^-5	4,9E+10	257333333	5,3E-03
1200-2	6	185	224	229	32	37	41	212,7	10^-7	36,7	10^-5	8,5E+10	146666667	1,7E-03
	7	129	140	156	4	3	4	141,7	10^-7	3,7	10^0	5,7E+10	147	2,6E-09
	8	116	117	125	2	3	1	119,3	10^-7	2,0	10^0	4,8E+10	80	1,7E-09
0 s	9	87	114	95	3	2	0	98,7	10^-7	1,7	10^0	3,9E+10	67	1,7E-09
	10	124	181	135	3	2	1	146,7	10^-7	2,0	10^0	5,9E+10	80	1,4E-09
	11	148	162	173	1	0	0	161,0	10^-7	0,3	10^0	6,4E+10	13	2,1E-10
5 s	12	121	123	129	0	0	0	124,3	10^-7	0,0	10^0	5,0E+10	0	0,0E+00
	13	115	122	114	1	2	1	117,0	10^-7	1,3	10^0	4,7E+10	53	1,1E-09
	14	111	127	128	0	0	1	122,0	10^-7	0,3	10^0	4,9E+10	13	2,7E-10
10 s	15	100	109	105	4	3	0	104,7	10^-7	2,3	10^0	4,2E+10	93	2,2E-09

After more than 10 seconds there were no transformants.

Table 4. Number of transformants from the introgression study in BD413, generation 0 to 6. 100, 500 and 1300 µl of the undiluted bacterial DNA was plated on LBRK50/50 plates and 100 µl on a LBR50 plate. The number of transformants on each plate is listed under the respective volumes of amount of bacterial DNA plated.

	LBRK		
Generation (donor DNA sonicated for x seconds)	100 µl	500 µl	1300 µl
16.4.0 (5s)	7	-	-
16.4.1 (10s)	226	TNTC	TNTC
16.4.2 (10s)	>300	>600	TNTC
16.4.2 (60s)	10	62	57+
16.4.2 (180s)	3	13	20
16.4.3 (60s)	1	5	6
16.4.4 (60s)	34	134	286
16.4.5 (10s)	30	172	TNTC
16.4.5 (30s)	1	0	1
16.4.5 (60s)	0	0	0
16.4.6 (10s)	347	TNTC	TNTC

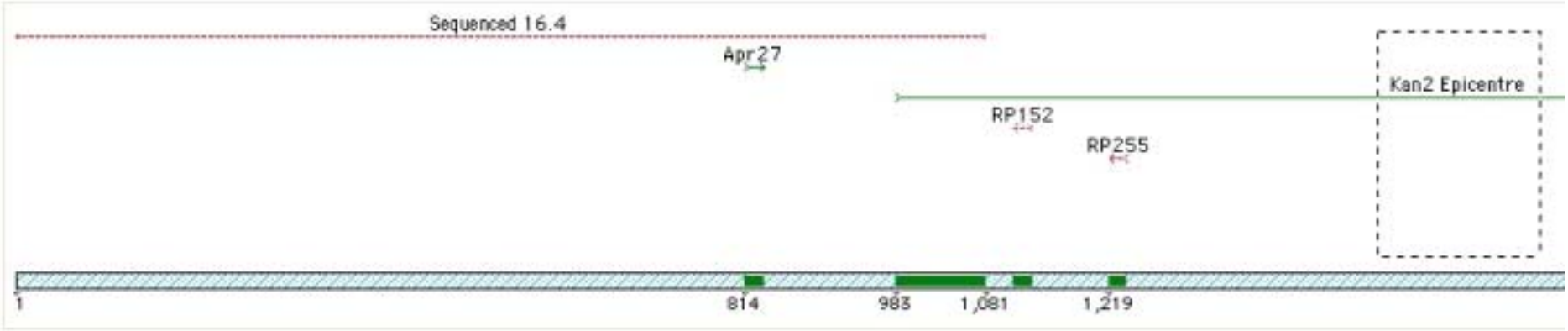
TNTC= too numerous to count, 0.9% NaCl was used as negative control, there was no growth on the LBRK50/50 plates with 0.9% NaCl and competent cells because A. baylyi strain BD413 is not resistant against kanamycin, unsonicated A. baylyi strain ADP1200-2 was used as positive control and there was lawn growth on these LBRK50/50 plates. Because the filters from the filter transformation were washed with 0.9% NaCl and undiluted bacterial DNA was plated, the total number of transformants is the number listed in the table multiplied by 20.

Table 5. Number of transformants from the introgression study in ADP7021, generation 0 to 1. 100, 500 and 1300 µl of the undiluted bacterial DNA was plated on LBRK50/50 plates and 100 µl on a LBR50 plate. The number of transformants on each plate is listed under the respective volumes of amount of bacterial DNA plated.

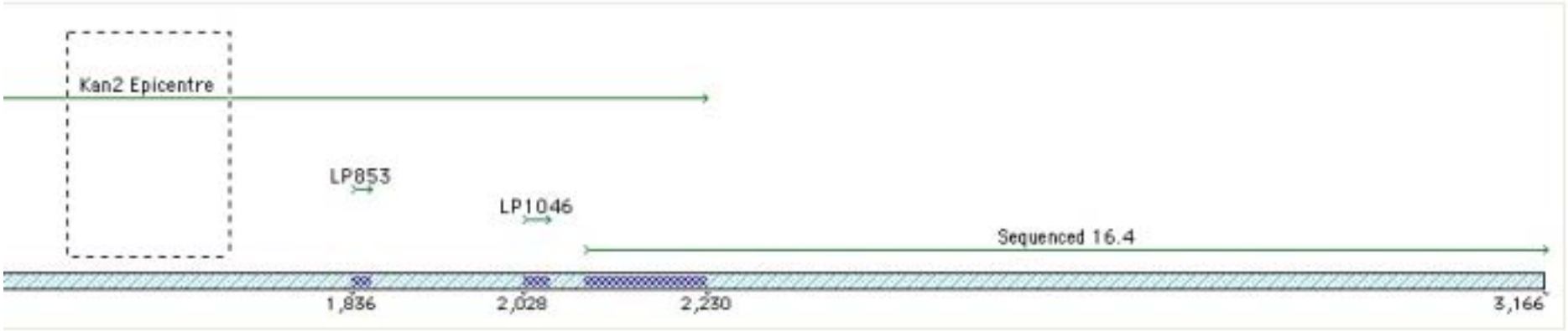
	LBSSK		
Generation (donor DNA sonicated for x seconds)	100 µl	500 µl	1300 µl
M16.4.0 (5s)	3	6	6
M16.4.0 (10s)	0	0	0
MS16.4.1.1 (5s)	0	1	0
MS16.4.1.1 (10s)	0	0	0
MS16.4.1.2 (5s)	0	2	0
MS16.4.1.2 (10s)	0	0	0
MS16.4.1.3 (5s)	0	0	0
MS16.4.1.3 (10s)	0	0	0
MS16.4.1.4 (5s)	0	0	0
MS16.4.1.4 (10s)	0	0	0
MS16.4.1.5 (5s)	0	0	2
MS16.4.1.5 (10s)	0	0	0

0.9% NaCl was used as negative control, there was no growth on the LBSSK50/50 plates with 0.9% NaCl and competent cells because A. baylyi strain ADP7021 is not resistant against kanamycin. Because the filters from the filter transformation were washed with 0.9% NaCl and undiluted bacterial DNA was plated, the total number of transformants is the number listed in the table multiplied by 20.

Figure 1. Map of sequencing primers. Three primers used for sequencing transformants of *A.* sp. strain 16.4 are not shown in the map. Primer Apr25 is designed to bind previous sequenced *A.* sp. strain 16.4 sequence immediately upstream of the *npt*II insertion. Primer RW20 and FW24 bind around 6000 nucleotides downstream and upstream.



Primers binding to the *npt*II gene and primer binding downstream for the *npt*II gene.



Primers binding to the nptII gene.