Sabrina Hammerlindl, BSc.

Development of a Flp/*frt* based integration system for *Lactobacillus plantarum*

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Begutachter: Univ.-Prof. Dipl.-Ing. Dr.techn. Helmut Schwab
Betreuerin: Mag.rer.nat. Sandra Nußbaumer

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Abstract

Lactic acid bacteria (LAB), like *Lactobacillus plantarum,* play an important role in the fermentation of food products and preservation of ensiled forages. They act as bio-preservatives, prevent microbial spoilage of the food products by acidification and produce inhibitory compounds.

One aim of this thesis was to transform and integrate single-stranded DNA (ssDNA) of the linear integration cassette 5´3´cbh_CAT into the genome of *L. plantarum*. This cassette consists of the homologous region of the *conjugated bile acid hydrolase* gene (5´cbh and 3´cbh) for the recombination event and the *chloramphenicol acetyl transferase* cassette (*CAT*) which was used as a selection marker. In an attempt to protect the ssDNA from nucleolytic degradation it was loaded with the single-stranded DNA binding proteins (SSB) and RecA prior to transformation into the *L. plantarum* cells. Therefore the genes of SSB and RecA, isolated from the *L. plantarum* CD033*,* were heterologously expressed in *E. coli* BL21DE3. The proteins were tagged with a N-terminally His_6-tag (SSBnHis and RecAnHis) and purified using a Ni-NTA sepharose column. The DNA binding activity of the expressed and purified SSBnHis and RecAnHis were investigated by gel shift assays. The integration of linear ssDNA loaded with SSBnHis and RecAnHis was investigated and compared to linear ssDNA without DNA binding proteins and linear dsDNA.

Another aim of this thesis was to develop a vector-free integration system based on the *S. cervisiae* Flp/*frt* recombination system using a linear cassette. This linear integration cassette consists of the homologous regions of the *conjugated bile acid hydrolase* gene (*cbh)*, the cellulase gene (*celAocES,* modified endocellulase from *Sorangium cellulosum*) under the control of the *efp* promoter (*Lactobacillus buchneri* CD034 *elongation factor P* promoter), a terminator sequence (*Lactobacillus* casei L-lactat dehydrogenase terminator, T_{ldh}), the *CAT* cassette and the *frt*-sites for the Flp/*frt* recombination system The recombination locus *cbh* is a non-essential gene for the *L. plantarum* strain and is therefore often used as recombination site for chromosomal integration. This cassette was generated, transformed and integrated as linear dsDNA which resulted in one chloramphenicol resistant colony. The correct integration into the genome of *L. plantarum* 3NSH was confirmed by PCR and sequence analysis.

Zusammenfassung

Milchsäurebakterien (LAB), wie *Lactobacillus plantarum*, spielen eine wichtige Rolle bei der Fermentation von Lebensmitteln und der Silierung von Futtermittel. Sie agieren als Bio-Konservierungsmittel, verhindern mikrobiellen Verderb der Lebensmittel durch Ansäuerung und produzieren hemmende Verbindungen.

Ein Ziel dieses Projektes war die Transformation und Integration von Einzelstrang-DNA (ssDNA) der linearen Integrationskassette 5´3´cbh_CAT in das Genom von *L. plantarum*. Diese Kassette besteht aus den homologen Region des *konjugierte Gallensäure Hydrolase* Gens (5´*cbh* und *3´cbh*), die für den Rekombinationsevent verwendet wurden und der *Chloramphenicol Acetyltransferase* (*CAT*) Kassette, die als Selektionsmarker diente. Um die ssDNA während der Transformation vor enzymatischem Abbau zu schützen wurde diese vor der Transformation mit den Einzelstrang-bindende Proteinen (SSB) und RecA beladen. Dafür wurden die aus *L. plantarum* stammenden Proteine in *E. coli* BL21DE3 heterolog exprimiert und mit Hilfe eines N-terminalen His_6-Tags (SSBnHis und RecAnHis) und einer Ni-NTA Sepharose Säule aufgereinigt. Die DNA Bindungseigenschaften der gereinigten Proteine wurde mittels Gel-Shift-Assay untersucht. Die Integration lineare ssDNA beladen mit SSBnHis und RecAnHis wurde untersucht und mit der Integration von unbeladener lineare ssDNA und linearer Doppelstrang DNA (dsDNA) verglichen.

Ein weiteres Ziel dieser Arbeit war es ein vektorfreies Integrationssystem zu entwickeln, dass auf dem Flp/*frt* Rekombinationssystem von *S. cervisiae* basiert. Die lineare Integrationskassette beinhaltet die homologen Regionen des konjugierten Gallensäure Hydrolase Gens (*cbh*), das Zellulase Gen (*celAocES,* modifizierte Endozellulase von *Sorangium cellulosum*) unter der Kontrolle des *efp* Promoters (*Lactobacillus buchneri* CD034 *Elongationsfaktor P*), einer Terminatorsequenz (*Lactobacillus casei L-laktat Dehydrogenase Terminator,* T_{ldh}), der *CAT* Kassette und den *frt*-Sequenzen für das Flp/*frt* Rekombinationsystem. Der Integrationssort, *cbh,* ist ein nicht–essentielles Gen für *L. plantarum* und wird deshalb oft als Rekombinationsstelle verwendet. Diese Kassette wurde als lineare dsDNA in *L. plantarum* transformiert und integriert. Dabei wurde eine einzelne chloramphenicol-resistente Kolonie erhalten. Die korrekte Integration in das Genom von *L. plantarum* 3NSH dieses Klons, wurde mittels PCR und Sequenzanalyse überprüft und bestätigt.

Table of Content

1 Introduction

1.1 *Lactobacillus plantarum*

Lactobacillus plantarum belongs to the lactic acid bacteria (LAB) and is an anaerobic but aero-tolerant, non-pathogenic, gram-positive, rod-shaped bacterium. It can be found in many different environmental niches as in vegetables, meat, dairy products and plant fermentations. *L. plantarum* is also a natural inhabitant of the mammal gastrointestinal tract [1]. The reason of this high habitat flexibility could be explained with the size of the genome and the responsible genes to utilize different types of carbohydrates. The genome of *L. plantarum* is the largest known among LAB and the strain *L. plantarum* strain WCFS1 has been fully sequenced by Kleerebezem et al., 2003 [2]. The size of the genome is around 3.3 megabases containing more than 3000 protein-encoding genes and has a G+C content around 44 % [3]. *L. plantarum* strain WCFS1 became one of the model strains for lactic acid bacteria (LAB) research [2].

L. plantarum belongs to the facultative heterofermentative LAB. This means it can degrade carbohydrates either by the glycolysis or the phosphoketolase pathway. The homofermentative LAB (glycolysis pathway) produce lactic acid as main product and the heterofermentative LAB (phosphoketolase pathway) produce equal amounts of carbon dioxide, ethanol and lactic acid [4].

The production of high amounts of lactic acid is the basis of food preservation. Different *L. plantarum* strains showed to be highly advantageous when used as inoculants for industrial grass and maize silages [5]. For that reason a lot of research and genetically manipulation of this bacterium was and will be done to enhance its fermentation profile [6].

1.2 Silage

Silage is the product from a process called silaging, ensiling or ensilage. Thereby, the water-containing material (grass, corn, clover-like forage, field beans, oats, beet leaves, etc.) is usually chopped to a silo and airtight covered. Lactic acid bacteria convert the water-soluble carbohydrates into organic acids (lactic acid) under anaerobic conditions. The pH decreases and growth of spoilage bacteria is inhibited. The silage is preserved as long as it is not exposed to air. Silage can be fed to cud-chewing animals such as cattle and sheep or used as a biofuel feedstock for anaerobic digesters [4].

The ensiling process can be divided into four phases: the aerobic phase (I), fermentation phase (II), the storage phase (III) and the feed-out phase (IV). In the first phase the air is still present between the forages layers and the pH is still neutral, which results in aerobic microbial activity until the air is depleted (takes a few hours). In the fermentation phase the growth of lactic acid bacteria is dominant and the production of lactic acid and other acids decrease the pH. This phase lasts for two weeks. The third phase is the stable phase of the ensiling process. In the last phase the silage is opened and fed to the animals. Thereby the fermented forage is re-exposed to air and aerobic microbial activity may get started [7].

Treatment with hydrolytic enzymes like cellulase or amylase can enhance the fermentation during the ensiling process and can increase the feed efficiency and thus the growth of beef bulls [8]. Furthermore, the addition of enzymes could improve the silage quality of forages with low sugar and/or high protein concentrations that usually are difficult to ensile [9].

1.3 Single-stranded DNA binding protein (SSB)

The SSB protein of *E. coli* plays a very important role for the DNA metabolism, like replication, repair and recombination. In *E. coli* the SSB monomer has 177 amino acids and a molecular weight of 19 kDa. It consists of two important parts, the N-terminal domain, which main structural motives are α-helices and β-sheets and the C-terminal domain, which mainly consists of loop structures. The N-terminal region contains the DNA binding motive also called the OB- (oligonucleotide binding) fold, figure 1 B) and oligomerization [10-12]. This OB-fold is a common ssDNA binding motif and was first discovered by Murzin, 1993 [13]. This motif contains five β-sheets forming an antiparallel β-barrel and an α-helix at the end (figure 1 A). The C-terminal region is not involved in the DNA binding or oligomerization but probably responsible for interactions with other proteins [14]. The end region of the C-terminus consists of a, ten amino acids, acidic tail, which can increase the affinity of the SSB protein for ssDNA when the tail is mutated or removed [15].

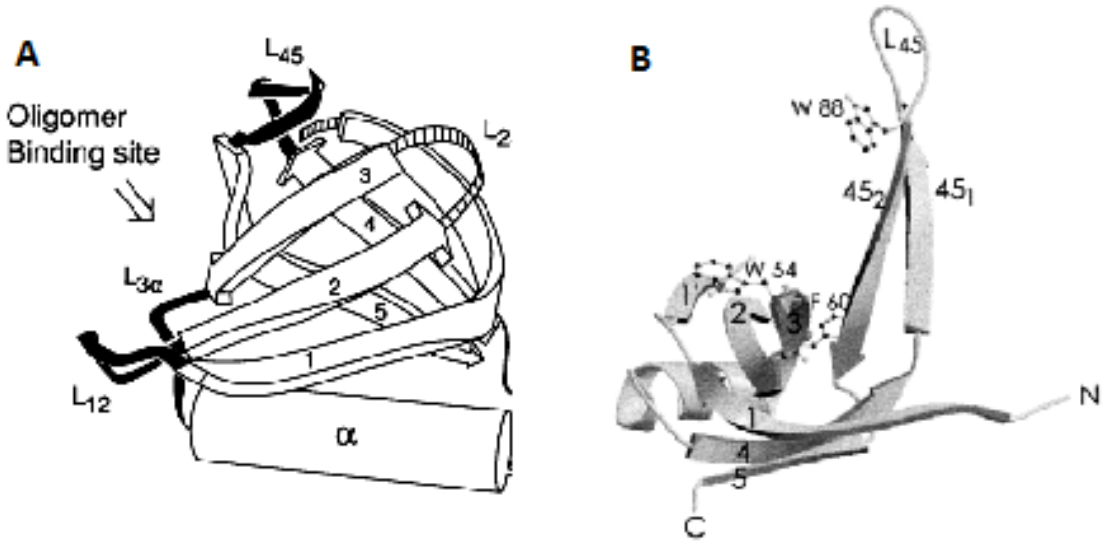

Figure 1*: **Scheme of the OB-fold.** (A) Discovered from Murzin, 1993 [13]. The numbered arrows are the β-sheets, the cylinder represents an α-helix and the loops are shown as ribbon. The three black loops describe the residues involved in the oligomer binding. (B) The scheme of the E.coli SSB monomer also consists of five β-sheets, one α-helix and the loop L45 which might also be involved in the oligomer binding [10].*

The active SSB protein is a homotetramer. Two different crystal structures were discovered by Raghunathan et al., 1997, a tetramer formed by two L_{45} loops and a tetramer formed by two six stranded β-sheets (figure 2) [10].

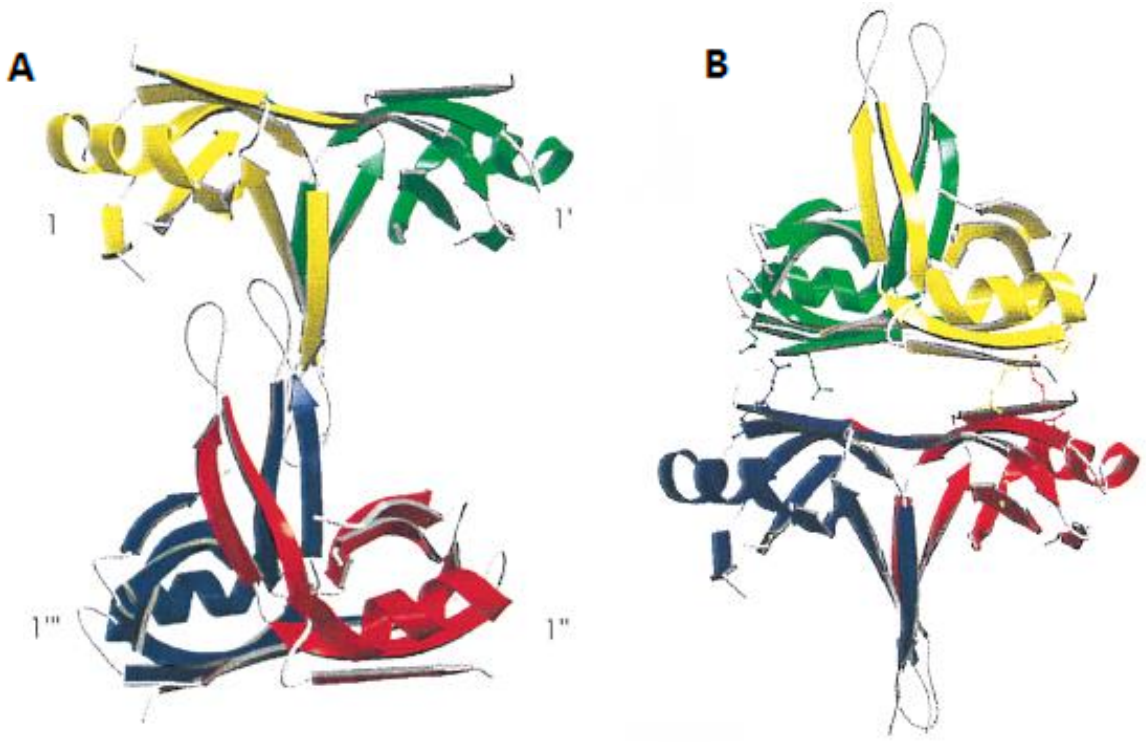

Figure 2*: Scheme of the two different crystal structures of the SSB tetramer.* *(A) L_{45}-mediated tetramer, 1 is the first monomer and 1´, 1´´ and 1´´´ the second, third and fourth monomer. (B) Six stranded β-sheet mediated tetramer. Adapted from [10].*

Studies showed that the six-stranded β-sheet configuration of the SSB tetramer represents the structure in solution. The functional SSB tetramer binds tightly to the ssDNA and covers a stretch of 8 to 12 nucleotides. The binding to the ssDNA prevents premature annealing, protects the DNA from nuclease activity and removes secondary structures to allow other enzymes to function effectively. The SSB is one of the most used proteins for studying protein-nucleic acid interactions [16]. In the *L. plantarum* JDM1 strain the SSB monomer has a calculated size of 21.7 kDa (Accession number C6VIH7) and presumably has the same functions like in *E. coli*.

1.4 RecA protein

The E. *coli* protein RecA plays an important role in the repair and maintenance of DNA. It has many different tasks related to DNA repair, like the regulation of the activity of the LexA- and the λ –repressors (figure 3). There it acts as a co-protease in the autocatalytic cleavage of the LexA repressor during the SOS response [17]. In the homologous recombination mechanism RecA binds strongly and in long clusters to ssDNA to form a nucleoprotein filament. It has more than one DNA binding sites which enables simultaneously binding of ssDNA and dsDNA. The RecA-DNA filament searches for sequence similarity along the dsDNA. This causes stretching of the DNA duplex which facilitates the exchange of strands between two recombining DNA double helices. After the synapsis event, a process called branch migration begins in the heteroduplex region. In this process an unpaired region of one of the single strands displaces a paired region of the other single strand, moving the branch point without changing the total number of base pairs [18].

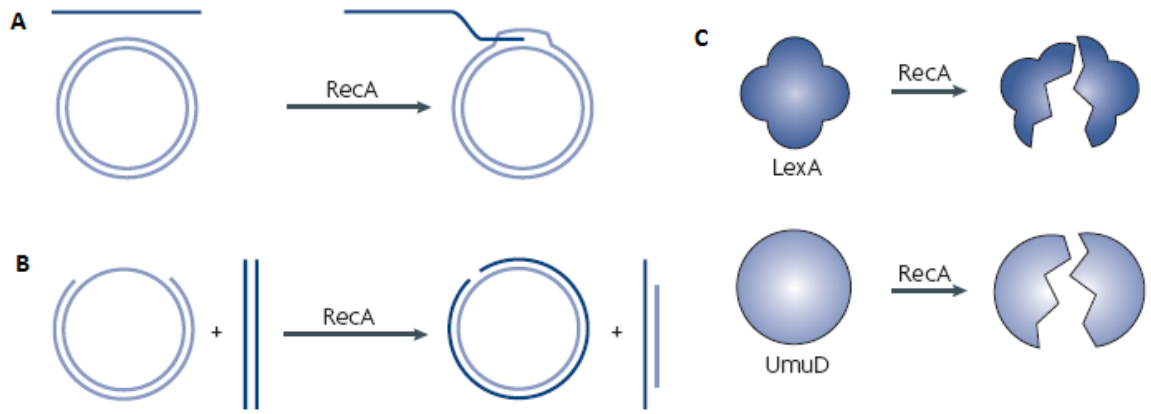

Figure 3: ***Examples for the RecA activity in E.coli.*** *(A) strand-invasion reaction causing D-loop formation, (B) four-strand-exchange reaction and (C) RecA coprotease function. RecA facilitates the autocatalytic cleavage of LexA, UmuD and other proteins [19].*

The RecA monomer of *E. coli* has 352 amino acids and a molecular weight of 38 kDa. The structure of this protein was first discovered from Story, Weber, & Steitz, 1992 [20]. The RecA protein consists of a large core domain (RecA-fold), an N-terminal and a C-terminal domain (figure 4) [21]. The N-terminal domain is involved in the filament formation process and the C-terminal domain is also involved in the protein-protein interaction and probably responsible for the coprotease activity [22].

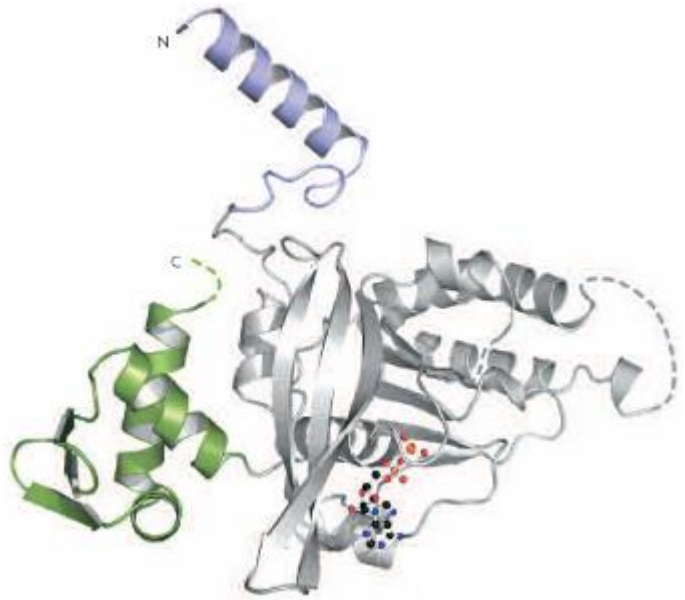

Figure 4: ***Scheme of the E. coli RecA protein.*** *The protein consists of a large core domain (grey), an N-terminal (blue) and a C-terminal (green) domain [19].*

The in *L. plantarum* WCFS1 strain occurring RecA protein has a calculated size of 40.7 kDa (Accession number Q88UZ4) and presumably the same functions like in *E. coli*.

1.5 Conjugated bile acid hydrolase (Cbh)

Bile acids are common in the intestinal tract of mammal and play an essential role in the lipid digestion. They act as emulsifiers for the lipids and lipid-soluble vitamins. Bile acids are synthesized in the liver and get conjugated as N-acyl amidat with taurine or glycine. Bacteria like *L. plantarum* and other intestinal lactobacilli produce the conjugated bile acid hydrolyse (Cbh) in the intestinal tract. Cbh catalyse the deconjugation of the conjugated bile acids. The bile acid is then transported back into the liver. It is proven that *cbh* is not an essential gene for *L. plantarum* which is the reason why this gene is used as the target gene for stable integration into the *L. plantarum* genome via homologous recombination [23, 24].

1.6 Homologous recombination

Homologous recombination occurs in all organisms and it is a genetic tool to achieve chromosomal integration of desired genes. It is also a repair system for double strand breaks in bacteria and produces new combinations of DNA sequences during meiosis in eukaryotic cells.

There are two different techniques to integrate a vector into a chromosome: a one-step single-crossover (the Campbell-like integration) and a one- or two-step double-crossover recombination event. During the Campbell-like integration a vector DNA is inserted into the host chromosome. Therefore a non-replicating vector, containing homologous regions to the target genome is needed. The integration efficiency depends on the size of the homologous sequence and of the recombination efficiency of the host. This type of recombination can lead to a multi-copy integration of the vector DNA into the same locus of the chromosome and it shows instability. In figure 5 a typically single-crossover event is illustrated [25].

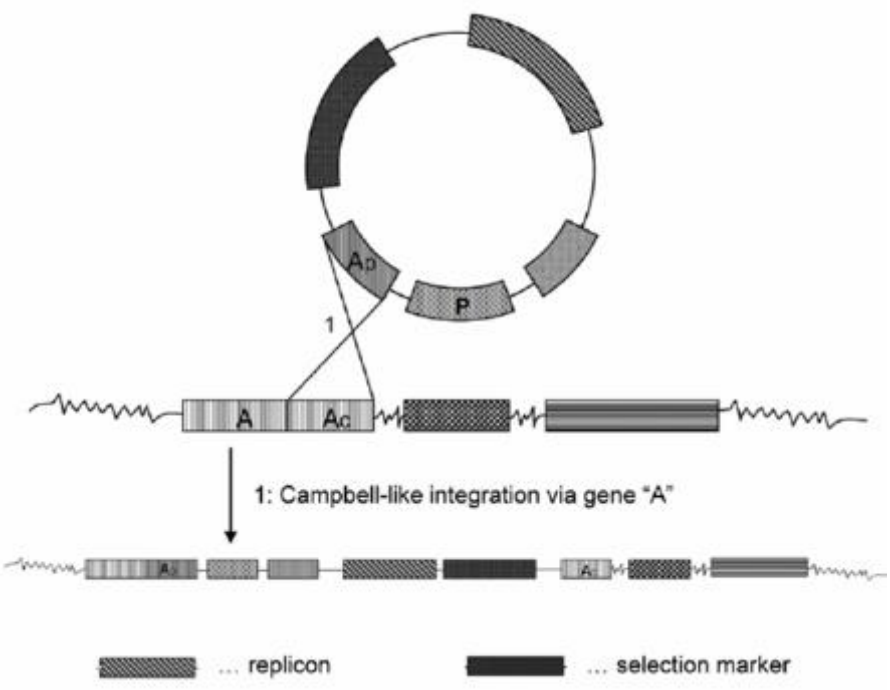

Figure 5: Illustration of the Campbell-like integration. *Thereby the homologous region "A" (Ap and Ac) are used. The integration vector contains also the gene of interest "P", a non-functional replicon and a food-grade selection marker. Due to the use of a food-grade selection marker the whole vector can be stable integrated into the host chromosome. One homologous region is adequate for Campbell-like integration [25].*

In case of the double-crossover recombination event, the integration vector contains two, physically closely linked, homologous regions. As mentioned above there are two types of double-crossover events, the one-step and the two-step gene replacement. During the one-step double-crossover event the recombination reaction happens simultaneously on both recombination sites. The gene of interest on the integration vector replaces the gene on the host chromosome (see figure 6) [25].

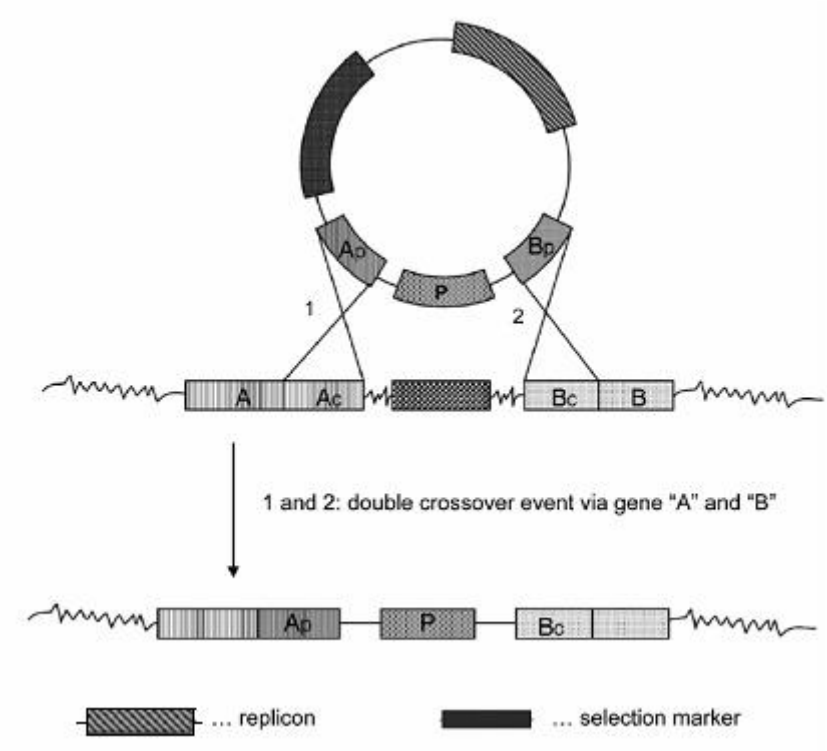

Figure 6: Illustration of the one-step double-crossover event. *Thereby the homologous region "A" (Ap and Ac) and "B" (Bp and Bc) are used. The gene of interest "P" replaces the gene on the chromosome in one step [25].*

While in the two-step double-crossover event two different reactions occur. In the first step the complete vector DNA is integrated into the host genome via one homologous site (Campbell-like integration). In the next step a loop-out deletion event either via the homologous region of gene "A" or gene "B" will start. If the second crossover occurs via the region "A" the phenotype of the wildtyp will be restored and if the loop-out deletion happens via the region "B" the homologous recombination of the target gene will be achieved (see figure 7). The second step can be induced by using a thermosensitive system or by reduction of the selection pressure [25].

The two-step double-crossover event was first illustrated in *B. subtilis* and was later on optimized for *L.* lactis [26].

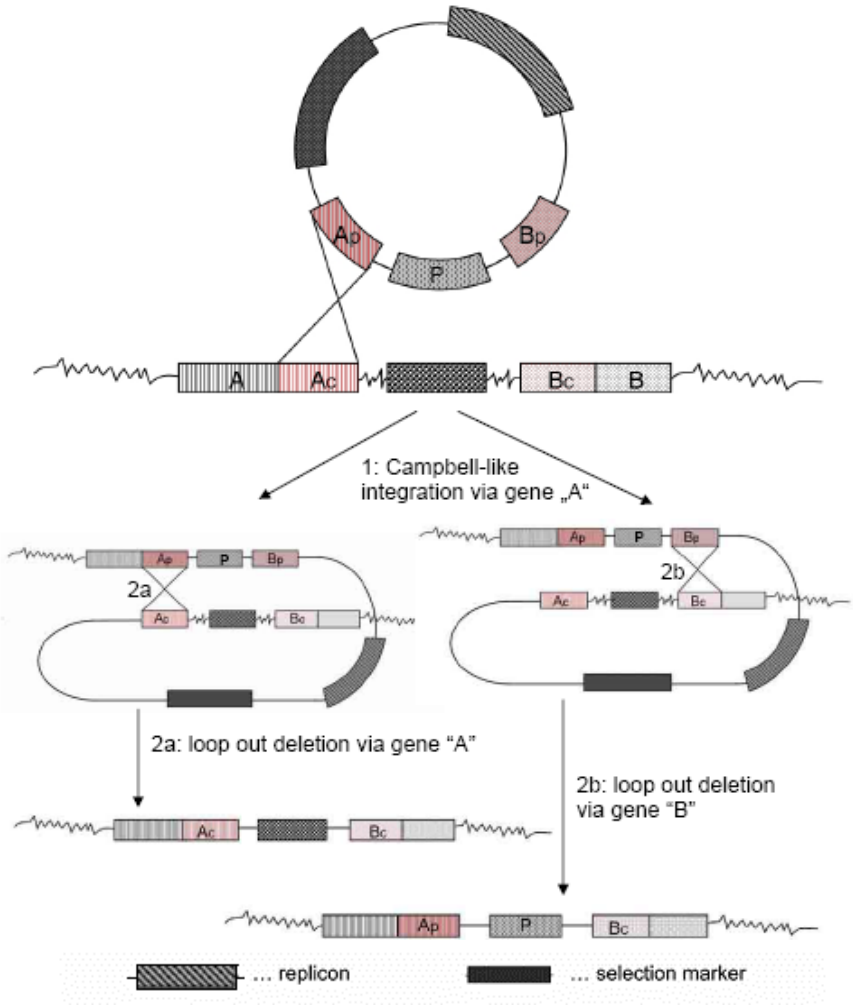

Figure 7: Illustration of the two-step double-crossover event. *In the first step a Campbell-like integration via gene “S” occur (1). In the second step a loop-out deletion event either via the homologous region of gene “A” (2a) or gene “B” (2b) occurs. Two different phenotypes can be generated: the wildtyp via “A” and the desired phenotype with the gene of interest via “B” [26].*

1.7 Site-specific recombination technology

. Site-specific recombination moves specialized nucleotide sequences, between non-homologous sites within a genome. The movement can occur between two different positions in a single chromosome, as well as between two different chromosomes. Site-specific recombinases, like the Flippase (Flp) or Cre recombinase, perform the rearrangement of DNA segments by recognizing and binding to specific nucleotide sequences, where they cleave the DNA backbone, exchange the two DNA helices involved and seal the cleaved DNA strands [27].

1.7.1 Flp/*frt* recombination system

This system comes originally from *S. cervisiae.* The yeast harbours a 2 µm plasmid (autonomous replicating), which contain the Flp (Flippase) recombinase and two Flp-recombinase target (*frt*) sites. The Flp protein has 423 amino acids and the *frt* sites have 48 base pairs (bp) with three 13 bp symmetry elements (a, b and c in figure 8) and an 8 bp core containing the *Xba*I site for the Flp cleavage. The *frt* sites can be organized in two different ways, inverted *frt* sites leads to inversion of the DNA sequences during the recombination process and direct oriented *frt* sites causes deletion of the DNA sequences (figure 9) [28, 29].

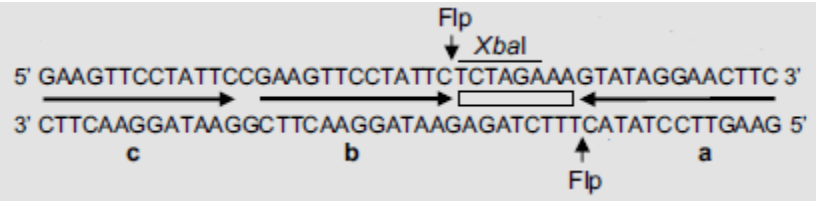

Figure 8: Sequence of the frt sites. *The horizontal arrows mark the 13 bp symmetry elements a, b and c. The box indicates the 8 bp core and the XbaI site is underlined. The vertical arrows indicate the Flp cleavage sites [29].*

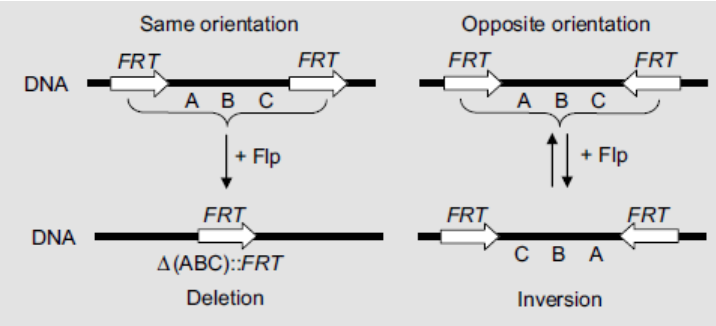

Figure 9: Flp recombination events. *frt sites in the same direction cause deletion and inverted frt sites cause inversion of the DNA sequences [29].*

The Flp/*frt* system has been studied excessively in a lot of organism and has demonstrated a high efficiency in gen manipulation. Beside this mutagenesis technique, the Flp/*frt* system is also adaptable for integration of heterologous DNA into the genome, removal of unwanted sequences and many other applications. An example of use for the Flp/*frt* recombination system is shown in the following figures. Figure 10 shows a sites-specific recombination for phytosensing in plants [30].

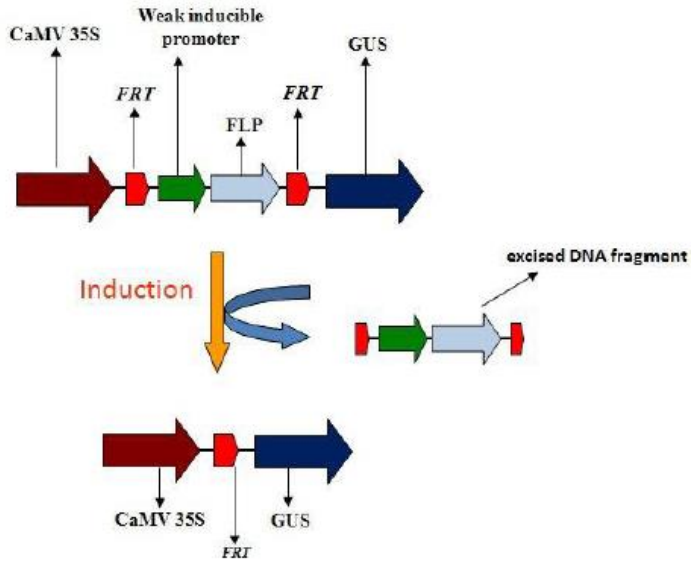

Figure 10: Scheme of the recombination system for the phytosensing. *CaMV 35S promoter is the promoter from Cauliflower Mosaic Virus. GUS indicates the β-glucuronidase. After induction of the inducible promoter the expressed Flp recombinase recognize the frt sites and will exclude the DNA sequence between them. The GUS reporter gene is under the control of the CaMV 35S promoter [30].*

Figure 11 shows the system for construction of unmarked bacterial strain carrying heterologous DNA [29].

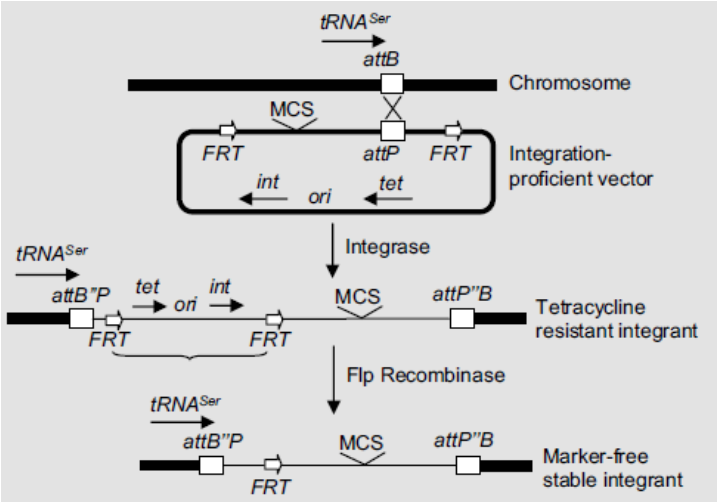

Figure 11: Scheme of the recombination system for the generation of a marker-free strain. *A non-replicative plasmid containing the tet gen for the tetracycline resistance, the int gene for the integrase, which catalyses the site-specific integration into the genome and the MSC (multiple cloning site, which represents the heterologous gen of interest) is used for the generation of a marker-free strain. The integration event occurs by the chromosomal attB sites and the plasmid-contained attP site. The expressed Flp recombinase excludes the undesired DNA sequences, including the antibiotic resistance marker [29].*

2 Objectives

Preservation of forages is the main emphasis of the ensilage process. The conversion of water soluble carbohydrates to organic acids, which is often done by lactic acid bacteria, results in a reduced pH value and therefore a toxic environment for spoilage bacteria. Genetic optimization of these lactic acid bacteria, like the recombinant expression of cellulases, is a valuable tool for improving the ensilage process. However, as part of the food chain, the optimized lactic acid bacteria must meet the requirements for food grade microorganisms. To satisfy these requirements the microorganisms are, for example, not allowed to carry any artificial resistance genes that often are an essential part of cloning strategies. Therefore the molecular strategies used to generate food grade microorganisms must include ways to overcome such obstacles. An example would be to utilize a site specific recombination system, like the Flp/*frt* recombination system, that can be used to remove the artificial resistance gene. Additionally, alternative strategies for the integration of genetic material into the lactic acid bacteria genome could improve the integration efficiency and facilitate genetic processing of these microorganisms. One such strategy is to transform the target genes as linear ssDNA. Using ssDNA could facilitate the strand invasion step of the recombination event which could lead to increased integration efficiency compared to integration of dsDNA. Therefore the aims of this thesis were:

1) Generation and transformation of a linear integration cassette containing the homologous regions of the conjugated bile acid hydrolase gene (*cbh)*, the cellulase gene (*celAocES,* modified endocellulase from *Sorangium cellulosum*), the *efp* promoter (*Lactobacillus buchneri* CD034 elongation factor P promoter), a terminator sequence (*L. casei L-lactat dehydrogenase terminator*, T_{ldh}), the *chloramphenicol acetyl transferase* (*CAT*) cassette and the *frt*-sites for the Flp/*frt* recombination system. The recombination system is required to remove the selection marker (*CAT*) in the genetically modified organism, which is necessary to meet the requirements of the food grade microorganisms.
2) Recombinant expression and purification of the His_6 tagged SSB and RecA from *L. plantarum* CD033.

3) Transformation of the short integration cassette 5´3´cbh_CAT (contains the homologous region of the *cbh* gene and the *CAT* cassette) as ssDNA loaded with SSB and RecA to compare the integration efficiency to linear ssDNA without DNA binding proteins and linear dsDNA.

3 Materials

All used chemicals were purchased from the following companies: Sigma-Aldrich, Roth, Fluka and Merck. Exceptions are mentioned separately. The restriction enzymes were from Thermo scientific.

3.1 Culture Media

3.1.1 Growth media

LB- media (lysogeny broth): 5 g/l yeast extract, 5 g/l NaCl and 10 g/l Tryptone
Components were dissolved in water and autoclaved by 121°C for 15 min.

MRS-media (for *Lactobacillus plantarum*): 10 g/l casein peptone, 8 g/l meat extract, 4 g/l yeast extract, 20 g/l D(+)-glucose, 2 g/l di-potassium hydrogen phosphate, 1 g/l Tween 80, 2 g/l di-ammonium hydrogen citrate, 5 g/L sodium acetate, 0,2 g/l magnesium sulphate, 0,04 g/L manganese sulphate.
Components were dissolved in water and autoclaved by 118°C for 15 min.

Media for agar-plates: 15 g/l Agar-Agar added to the LB- or MRS-media.

To get a selection media, the required amount of ampicillin, kanamycin or chloramphenicol was added to the media to get the final concentration of 100 µg/ml, 40 µg/ml and 10 µg/ml respectively.

3.1.2 Antibiotics

Ampicillin: Stock solution 100 mg/ml (in water)

Kanamycin: Stock solution 40 mg/ml (in water)

Chloramphenicol: Stock solution 10 mg/ml (in ethanol)

3.1.3 Media for the Congo red-assay

MRS-CMC agar-plates: 5 g/l CMC (carboxymethyl cellulose) added to the components of the MRS- agar, dissolved in water and autoclaved by 118 °C for 15 min.

LB-CMC agar-plates: 5 g/l CMC (carboxymethyl cellulose) added to the components of the LB- agar, dissolved in water and autoclaved by 121 °C for 15 min.

3.2 Standards for:

DNA: GeneRuler^{TM} DNA Ladder Mix 100-10000 bp (#SM0332, Thermo scientific)

1 kbp DNA ladder (Y014.1, Carl Roth)

Proteins: PageRuler prestained Protein Ladder (Thermo scientific)

3.3 Microorganism

Table 1: Bacterial strains used in this work

Name	**Application**	**Source**
E. coli XL1-Blue	Plasmid cloning	IMBT#923
E. coli BL21DE3	Protein expression	IMBT#1421
L. plantarum CD033	Integration of linear DNA	IMBT#6326
L. plantarum 3NSH	*L. plantarum* CD033 plasmid pCD033 free strain, integration of linear DNA	CD-Lab #197

3.4 Plasmids

Table 2: Plasmids used in this work

Name	Description	Source
pEfp_celAocES	Cm^r, Ap^r, P_{efp}, pUC19 containing origin from p256 and cellulase gene *celAocES*	CD-Lab Graz strain collection #127
pET28b(+)	Km^r, P_{T7}, His_6-tag	Novagen, Cat. No. 69258
pET28b_ssb	Km^r, P_{T7}, His_6-tags, containing *ssb*	CD-Lab, previous work of my own, project lab, not preserved
pINT	Cm^r, Ap^r, ori(pMB1), ori(pE194), Integration vector for *L. plantarum* containing the homologous regions of *cbh*	CD-Lab Graz strain collection #39
pINT_efp_celAocES	pINT backbone containing P_{efp} and the cellulase gene *celAocES*	CD-Lab Graz strain collection #113
pINT_efpshort1_celAocES	pINT backbone containing short version 1 of the P_{efp} and the cellulase gene *celAocES*	This thesis, not preserved
pINT_efpshort2_celAocES	pINT backbone containing short version 2 of the P_{efp} and the cellulase gene *celAocES*	This thesis, not preserved
pINT_terminator	Cm^r, Ap^r, ori(pMB1), ori(pE194), Integration vector for *L. plantarum* containing the homologous regions of *cbh* and the *L. casei* L-lactat dehydrogenase terminator (T_{ldh})	CD-Lab Graz strain collection #122

pINT_term_5´cbhnew	pINT_terminator backbone containing the *5´cbh* from pMS470_5´3´cbh_CAT	This thesis, not preserved
pINT_term_5´cbh_efp_celAocES	pINT_terminator backbone containing the *5´cbh* from pMS470_5´3´cbh_CAT, containing the promoter P_{efp} and the cellulase gene *celAocES*	This thesis, not preserved
pINT_term_5´cbh_efpshort1_celAocES	pINT_term_5´cbh_efp_celAocES backbone containing the short version 1 of the P_{efp} and the cellulase gene *celAocES*	This thesis, not preserved
pINT_term_5´cbh_efpshort2_celAocES.	pINT_term_5´cbh_efp_celAocES backbone containing the short version 2 of the P_{efp} and the cellulase gene *celAocES*	This thesis, not preserved
pJET1.2	Ap^r, P_{lacUV5}, *eco47IR,* P_{T7}	CloneJET™ PCR Cloning Kit (Thermo scientific)
pJET_frt_CAT	pJET1.2 backbone containing the *frt*-sites and *CAT* (*chloramphenicol acetyl transferase*)	This thesis, not preserved
pJET_frt_3´cbh	pJET1.2 backbone containing the *frt*-sites and the *3´cbh* from pMS470_5´3´cbh_CAT	This thesis, not preserved
pJET_fcf3c	pJET1.2 backbone containing the *frt*-sites, *CAT* (*chloramphenicol acetyl transferase*) and the *3´cbh* from pMS470_5´3´cbh_CAT	This thesis, not preserved
pJET_recA	pJET1.2 backbone containing the *recA* gene	This thesis, not preserved

pJET_5´cbh_efp_celAocES_fcf3c	pJET1.2 backbone containing the *5´cbh* from pMS470_5´3´cbh_CAT, promoter P_{efp}, the cellulase gene *celAocES*, the *frt*-sites and *CAT* (*chloramphenicol acetyl transferase*)	This thesis, IMBT strain collection #7418
pJET_5´cbh_efpshort2_celAocES_fcf3c	pJET_5´cbh_efp_celAocES_fcf3c backbone containing the short version 2 of the promoter P_{efp}	This thesis, IMBT strain collection #7419
pMS470Δ8	Ap^r, P_{tac}	IMBT strain collection #993
pMS470_linker	pMS470Δ8 (derivative), containing linker with the *Bgl*II restriction site	CD-Lab, previous work of my own, project lab, not preserved
pMS470_linker_recA	pMS470Δ8 (derivative), containing linker with the *Bgl*II restriction site and the *recA* gene	This thesis, not preserved
pMS470nHis	pMS470Δ8 (derivative), containing N-terminal His_6-tag	CD-Lab Graz strain collection #139
pMS470nHis_recA	pMS470Δ8 (derivative), containing N-terminal His_6-tag and the *recA* gene	This thesis, CD-Lab Graz strain collection #229
pMS470nHis_ssb	pMS470Δ8 (derivative), containing N-terminal His_6-tag and the *ssb* gene	This thesis, IMBT strain collection #7417
pMS470_ssb	pMS470Δ8 (derivative), containing the *ssb* gene	CD-Lab, previous work of my own, project lab, not preserved

pMS470_5´3´cbh_CAT	pMS470Δ8 (derivative), containing homologous region of the *conjugated bile acid hydrolase* gene (*cbh*, *5´cbh* and *3´cbh)* and *CAT* (*chloramphenicol acetyl transferase*) cassette	IMBT strain collection #7420, previous work of my own, project lab

Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; P_{LacUV5}, Modified P_{lac} for expression of the *eco47IR* gene*;* P_{efp}, *L. buchneri* CD034 elongation factor P promoter, which contains remains of the gene encoding the ribosomal protein L27 (from *L. buchneri*) and remains of the *CAT* sequence; short version 1 of the P_{efp}, remains of the *CAT* sequence have been removed (64 bp); short version 2 of the P_{efp}, remains of the *CAT* sequence and the gene encoding the ribosomal protein L27 (from *L. buchneri*) have been removed (129 bp). Sequences of the *efp* promoter versions are shown in table 25 in the appendix.

3.5 Kits

Genomic DNA Extraction: Easy-DNA™ gDNA Purification Kit (Life Technologies)

DNeasy® Blood&Tissue Kit (Qiagen)

DNA Purification: Wizard® SV Gel and PCR Clean-Up System (Promega)

GeneJET PCR Purification Kit (Thermo Scientific)

Plasmid Extraction and Purification: GeneJET Plasmid Miniprep Kit (Thermo scientific)

PCR cloning: CloneJET™ PCR Cloning Kit (Thermo scientific)

3.6 Buffer

3.6.1 SDS-PAGE:

20x MES-Buffer for NuPAGE® SDS PAGE Gel System (Life Technologies):

195.2 g MES

121.2 g Tris

20 g SDS

6 g EDTA

pH 7.3

Dissolve in 800 ml H_2O and fill to 1 l.

1x MES-Buffer:

50 ml 20x MES-Buffer

Fill to 1 l.

10x Running Buffer for self-made polyacrylamid gels:

60 g Tris

288 g glycine

20 g SDS

Dissolve in 1.5 l H_2O, set pH to 8.6 and fill to 2 l.

Coomassie Brilliant Blue staining solution

100 ml methanol

20 ml acetic acid

0.1 g Brilliant blue G 250

80 ml H_2O

3.6.2 Westernblot

20x Transfer Buffer:

10.2 g Bicine

2 g Bis-Tris

0.75 g EDTA

Dissolve in 125 ml H_2O.

1x Transfer Buffer:

50 ml 20x transfer buffer

1 ml NuPAGE® Antioxidant

100 ml methanol

Fill to 1 l.

10x TBS:

80.6 g NaCl

2 g KCl

60.6 g Tris

Dissolve in 800 ml, set pH to 8.0 and fill up to 1 l.

3.6.3 Protein purification

Lysis Buffer:

5 mM imidazole

50 mM Na-phosphate

300 mM NaCl

Dissolve in 800 ml, set pH to 8.0 and fill up to 1 l.

Wash Buffer:

30 mM or 40 mM imidazole

50 mM Na-phosphate

300 mM NaCl

Dissolve in 800 ml, set pH to 8.0 and fill up to 1 l.

Elution Buffer:

500 mM imidazole

50 mM Na-phosphate

300 mM NaCl

Dissolve in 800 ml, set pH to 8.0 and fill up to 1 l.

SSB Storage Buffer [31]:

20 mM Tris

1 mM EDTA

500 mM NaCl

50 % glycerine

Dissolve in 800 ml and autoclave, add 10 mM β-Mercaptoethanol, set pH to 8.0 and fill up to 1 l and sterile filtered.

RecA Storage Buffer [32]:

20 mM Tris

10 % glycerine

Dissolve in 800 ml and autoclave, add 1 mM DTT, set pH to 7.5 and fill up to 1 l and sterile filtered.

ADH Buffer (kindly provided by Zalina Magomedova):

0.2 M NaCl

20 mM Tris

Dissolve in 800 ml and autoclaved, set pH to 8.0 and fill up to 1 l.

3.6.4 DNA loading assay

RecA assay Buffer [33]:

20 mM Tris

50 mM KCl

10 mM $MgCl_2$

Dissolve in 800 ml and autoclave, set pH to 7.4 and fill up to 1 l and sterile filtered.

Loading dye:

0.05 % bromophenol blue

40 % sucrose

Dissolve in 1 ml.

3.7 Primer

Table 3: Primer for the construction the recA amplification

Name	Sequence	Primer#
recA-*Nde*I_fwd	5'-GGTACAT-ATGGCTGATGCACGGA-3'	166
recA__ΔStop*Hind*IIIrev	5'-GCGC-AAGCTTTTTTTCGGTTGG-3'	137

Table 4: Primer for the construction of the Flp/frt based integration cassette

Name	Sequence	Primer#
efpshort1_*Sac*I_fwd	5'-GCATGAGCTC-GAAAAGGACGCGACAAG-3'	189
efpshort2_*Sac*I_fwd	5'-GCTCGAGCTC-GCACAATAGTTTAATTAAAGAGG-3'	190
*Bgl*II_efp_rev	5'-CCGAGATCT-GATATGCCTCCTAAATGTAATG-3'	188
*Xho*I_frt_CAT_fwd	5'-CTCGAG-gaagttcctatactttctagagaataggaacttc-CCTCGCCGGCAATAGTT-3'	180
*Spa*HI-*Bam*HI-CAT_rev	5'-GCATGCCGGATCC-AATGTCGGCATAGCGTG-3'	181
*Spa*HI-frt-3´cbh_fwd	5'-GCATGC-gaagttcctatactttctagagaataggaacttc-GGTGTGTTAACAAAC-3'	182
*Hind*III-*Eco*RI-3´cbh-rev	5'-AAGCTTGAATTC-TAAAAGAGGAAATCAACTG-3'	183
5'cbh552_*Eco*RI_fwd	5'-GATGATGAATTC-TTGGCTCTTAAAGTGTTTG-3'	122

Table 5: Primer for the verification of correct integration

Name	Sequence	Primer#
CAT_rev	5'-CAGAATGATGTACCTGTAAAGATAGCGGTA-3'	30
CAT_fwd	5'-CCAACAAACGACTTTTAGTATAACCACAGA-3'	31
celAopt_rev	5'-CTGCTGCAGTTATTCAGCAA-3'	58
chrom_cbh_rev	5'-TAACAGCAATGCCAGCCA-3'	108
chrom_cbh_fwd2	5'-TTACGAGGCCAACGCTAATG-3'	109
5'cbh552_rev	5'-GTCGACGGTACCAGAGCTCATTCCTGTTTATCAGCAACCA-3'	123
3'cbh522_fwd	5'-CTGGTACCGTCGACCTGCAGGGTGTGTTAACAAACAATCCT-3'	124
chrom_cbh_rev2	5'-CCCAACTTACCATCGAGC-3'	167
efpshort2_SacI_fwd	5'-GCTCGAGCTC-GCACAATAGTTTAATTAAAGAGG-3'	190

Table 6: Primer for the construction of linear ssDNA and dsDNA

Name	Sequence	Primer#
5'cbh_plantplant_fwd	5'-TTGGCTCTTAAAGTGTTTG-3'	138
3'cbh_linear_rev	5'-AGAGGAAATCAACTGATAAC-3'	139

4 Methods

4.1 General methods

4.1.1 Cultivation of microorganism

E. coli was grown over night at 37°C on LB agar or in LB bouillon supplemented with 100 µg/ml ampicillin or 40 µg/ml kanamycin for plasmid selection and maintenance. *L. plantarum* was grown under anaerobe conditions at 37°C for one to three days on MRS agar or in MRS bouillon supplemented with 10 µg/ml chloramphenicol, if required.

4.1.2 Preparation of plasmid and genomic DNA

Plasmid DNA was prepared using the GeneJET Plasmid Miniprep Kit (Thermo scientific). The corresponding *E. coli* strains were streaked out onto LB-Amp or LB-Kan agar plates and cultivated over night at 37°C. The bacterial culture was resuspended in 250 µl Resuspension Solution and lysed by adding 250 µl Lysis Solution. After inverting the tube 6 times, 350 µl Neutralization Solution were added to stop the lysis reaction. After inverting the tube 6 times the reaction mixture was centrifuged at 16100 x g for 20 minutes to pellet the cell debris and chromosomal DNA. The supernatant was transferred into the GeneJET spin column and centrifuged for one minute to bind the plasmid DNA. After two washing steps with 500 µl Wash Solution the plasmid DNA was eluted with 50 µl nuclease-free water after 2 minutes of incubation into a fresh tube.

Genomic DNA was isolated using the Easy-DNA™ gDNA Purification Kit (Life Technologies) according to the producer's protocol #3- Small amounts of cells, tissues or plants. The *L. plantarum* strain of interest was cultivated in an MRS or MRS-CM bouillon under anaerobe conditions at 37°C over night. The cells were pelleted and resuspended in 200 µl 1xPBS (phosphate buffered saline; 140 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO4, pH7.4). After addition of 350 µl Solution A and 5 µl Protein Degrader, the reaction mixture was incubated at 65°C for 30 minutes. Afterwards 150 µl Solution B were added to the tube and vortexed until the sample is viscous. After addition of 500 µl chloroform the sample was vortexed until it get homogenous and centrifuged at 16100 x g for 30 minutes at 4°C to separate the phases. The upper phase was carefully transferred into a fresh tube and 1 ml 100% ethanol (-20°C) was added to the DNA solution. After incubation for 30 minutes on ice the sample was centrifuged for 30 minutes at 4°C to pellet the DNA. Next, the pellet was washed with 80% ethanol (-20°C) and the residual ethanol was removed by pipetting. The DNA pellet was dried at room temperature for 5 minutes and resuspended in 100 µl nuclease-free water. After addition of 2 µl RNase (2 mg/ml) the sample was incubated over night at 4°C. After that the DNA was stored at 4°C.

Alternatively genomic DNA was isolated using the DNeasy® Blood&Tissue Kit (Qiagen) according to producer's protocol for gram- positive bacteria. Cells were harvested in a microcentrifuge tube by centrifugation for 10 min at 5000 x g. The resulting cell pellet was resuspended in 180 µl enzymatic lysis buffer (20 mM Tris·Cl, pH 8.0, 2 mM sodium EDTA,

1.2% Triton® X-100, 20 mg/ml lysozyme (Carl Roth) was added immediately before use) and incubated for 30 min at 37°C. Afterwards 25 µl proteinase K and 200 µl Buffer AL (without ethanol) were added, mix by vortexing and incubated at 56°C for 30 min. Next, 200 µl ethanol (100%) were added and mix by vortexing. This mixture was applied to the DNeasy Mini spin column placed in a 2 ml collection tube and centrifuged at 6000 x g for 1 min. The DNeasy Mini spin column was placed in a new 2 ml collection tube. Afterwards 500 µl Buffer AW1 were added and centrifuged for 1 min at 6000 x g. After centrifugation the DNeasy Mini spin column was placed in a new 2 ml collection tube, 500 µl Buffer AW2 were added and centrifuged for 3 min at 20,000 x g. The DNeasy Mini spin column was placed in a 1.5 ml microcentrifuge tube. 100 µl of nuclease-free water was added to the column, incubated for 1 minute at room temperature followed by centrifugation for 1 min at 6000 x g.

4.1.3 Polymerase chain reaction (PCR)

Standard PCR:

The DNA template for the PCR was obtained by plasmid isolation or genomic DNA extraction. The PCR was carried out according to the Phusion™ High-fidelity DNA Polymerase manual (2012, Thermo scientific) for a 50 µl reaction (table 7) or the DreamTaq Green PCR Master Mix (2012, Thermo scientific) for 25 µl reaction (table 8).

Table 7: Thermo cycler program and reaction mixture for the Phusion™ High-fidelity DNA Polymerase (Thermo scientific)

98°C	30 sec	
98°C	10 sec	25-35 cycles
Tm	30 sec	
72°C	30 sec/ kb	
72°C	5 min	
4°C	∞	

Component [µl]	
1	Template DNA (20-500 ng)
5	Primer forward (5 µM)
5	Primer reverse (5 µM)
10	5x Phusion HF Buffer
1	10 mM dNTP´s
0.2	Phusion polymerase (2 U/µl)
27.8	Sterile H_2O

Table 8: Thermo cycler program and reaction mixture of the DreamTaq Green PCR Master Mix (Thermo scientific)

95°C	1 min	
95°C	30 sec	25-40 cycles
Tm-5°C	30 sec	
72°C	1 min/kb	
72°C	5 min	
4°C	∞	

Component [µl]	
0.5	Template DNA (20-500ng)
2	Primer forward (10 µM)
2	Primer reverse (10 µM)
12.5	2x DreamTaq Green PCR Master Mix
8	Sterile H_2O

Colony PCR:

Colony PCR was used for the screening of positive clones. A positive clone contains the gene of interest, for example the *recA* gene in the pMS470_linker vector. Therefore, the gene specific primers (166 and 137, see table 3 primer list for details) were used to determine a positive clone. The Colony PCR was performed according to the DreamTaq DNA Polymerase (Thermo Scientific) manual. In table 9 an example of a PCR-mix and the thermo cycler program for the screening of the *recA* gene are shown.

Table 9: Thermo cycler program and reaction mixture for the Colony PCR

95°C	1 min	
95°C	30 sec	25 cycles
62°C	30 sec	
72°C	90 sec	
72°C	5 min	
4°C	∞	

Component [µl]	
	Single colony
2	Primer 166 (10 µM)
2	Primer 137(10 µM)
12.5	2x DreamTaq Green PCR Master Mix
8	Sterile H_2O

4.1.4 Agarose gel electrophoresis:

Agarose gel electrophoresis (1% agarose, Biozyme LE) was used to determine DNA concentration, to examine the quality of restriction experiments or PCR reactions (analytical gels) and to purify amplified DNA fragments (preparative gels). The agarose was dissolved in TAE buffer (40 mM TRIS, 20 mM Acetic acid, 1 mM EDTA) by heating in a microwave oven. To visualize the DNA 1% ethidium bromide (Carl Roth) was added and the gels were investigated under a UV-lamp. The electrophoresis for analytical gels was performed at 120 volt for 45 minutes and for preparative gels at 90 volt for 90 minutes (Bio-Rad PowerPac™ Basic). The concentration of the investigated DNA samples was determined by comparing the intensities of sample and standard bands. The sizes of the DNA fragments were estimated using the standard bands of the GeneRuler™ DNA ladder mix (Thermo scientific) or the 1 kbp DNA ladder (Carl Roth).

4.1.5 DNA purification from Agarose gel slices and PCR product purification

Gel slice preparation:

The DNA was separated by agarose gel electrophoresis based on size. The bands of interest were cut out and the DNA was extracted by using the Wizard®SV Gel and PCR Clean-Up System from Promega. The gel slice was incubated with Membrane Binding Solution at 65°C (10 µl per 10 mg gel slice) until the gel slice is completely dissolved. The mixture was transferred into a SV Minicolumn and centrifuged at 16100 x g for 1 minute to bind the DNA on the membrane. After two washing steps with Membrane Wash Solution the SV Minicolumn was transferred into a fresh tube. The DNA was eluted with 50 µl nuclease-free water after 1 minute incubation at room temperature.

PCR product preparation:

An equal volume of Membrane Binding Solution of the Wizard®SV Gel and PCR Clean-Up System (Promega) was added to the PCR amplification. The mixture was transferred into a Minicolumn and centrifuged at 16100 x g for 1 minute to bind the DNA on the membrane. After two washing steps with Membrane Wash Solution the Minicolumn was transferred into a fresh tube. The DNA was eluted with 50 µl nuclease-free water after 1 minute incubation at room temperature.

Alternatively the PCR products were purified by using the GenJET PCR Purification Kit (Thermo Scientific). The DNA was mixed 1:1 with Binding Buffer and was loaded onto the GeneJET purification column by centrifugation at 16100 x g for minute. After a washing step with the Wash Buffer the GeneJET purification column was transferred into a fresh tube. The DNA was eluted with 50 µl nuclease-free water.

4.1.6 CloneJET™ PCR Cloning Kit

The PCR cloning was performed according to the CloneJET PCR Cloning Kit #K1231, #K1232 (Thermo scientific) producer's protocol for blunt end cloning. The ligation reaction summarized in table 10 was set up on ice vortexed briefly and centrifuged for 3-5 s. Next the ligation mixture was incubated at room temperature for 30 minutes and cleaned using the Wizard® SV Gel and PCR Clean-Up System (Promega) (see 4.1.5). 5 µl of the resulting DNA was used for transformation into electrocompetent *E. coli* XL1-Blue (see 4.1.10).

Table 10: Reaction mix for CloneJET™ PCR Cloning

Volume	Component
10 µl	2X Reaction Buffer
5 µl	purified PCR product
1 µl	pJET1.2/blunt Cloning Vector (50 ng/µl)
3 µl	Water, nuclease-free
1 µl	T4 DNA Ligase
20 µl	Total volume

4.1.7 Restriction digest

The DNA was digested with the corresponding restriction enzymes, according to the manuals of Thermo scientific. In table 11 an example for the reaction mixture is shown.

Table 11: Reaction mix for the DNA digestion

Component [µl]	
16	Nuclease free Water
2	10x recommended Buffer
1	DNA (1µg)
1	Restriction enzyme (10 U)
20	Total volume

4.1.8 Ligation reactions

For the majority of the ligation reactions a vector:insert DNA ratio of 1:3 was used. Due to the similar sizes of the 5´cbh_efp_celAocES and the frt_CAT_frt_3´cbh constructs a 1:1 ratio was used for the ligation of these fragments.

Following formula was used to calculate the amount of insert DNA for a 1:3 vector:insert ratio.

$$\frac{\text{100 ng of vector x kb size of insert}}{\text{kb size of vector}} \times \frac{3}{1} = \text{ng of insert}$$

For a 20 µl ligation reaction, the appropriate amount of digested and purified insert DNA was mixed with 0.5 µl T4 ligase from Promega (3 U/µl), 2 µl 10x T4 ligase buffer and with 100 ng of digested, dephosphorylated and purified vector DNA. The ligation reaction was incubated for 3 hours at room temperature or at 8°C over night. The T4 ligase was then inactivated by heating to 70°C for 10 minutes.

Before electroporation the ligation reaction was desalted using micro-dialysis. The ligation reaction was carefully applied to the membrane (CME membrane, MF-Millipore Membrane, 0.025 µm and hydrophilic) floating on the surface of sterile tridestillated water. The desalination was carried out for 30 minutes at room temperature.

4.1.9 Electrocompetent *E. coli* cells

20 ml LB media were inoculated with *E. coli* BL21DE3 or *E. coli* XL1-Blue and incubated overnight at 37°C and 120 rpm. Main cultures (each 400 ml LB media) were inoculated to an optical density at 600 nm (OD_{600}) of 0.1 (measurement with Eppendorf BioPhotometer). The cells were grown at 37°C and 120 rpm until an OD_{600} of 0.7 to 0.8 was reached. The chilled cultures were harvested by centrifugation with 3220 x g and 4°C (Beckman Coulter Avanti-J 20 XP centrifuge). The cell pellets were washed twice with sterile distilled water (1st step with 250 ml, 2nd with 100 ml).

Afterwards the cells were washed in 40 ml 10% Glycerol. Finally, the cells were resuspended in 1-2 ml 10% glycerol and 80 µl aliquots were pipetted into fresh tubes. After freezing in liquid nitrogen, the cells were stored at - 80°C.

4.1.10 Transformation in *E. coli* by electroporation

An appropriate amount of the desalted ligation reaction (1-10 µl) was added to 40 µl electrocompetent cells. In order to transform the DNA, *E. coli* XL1-Blue or *E. coli* BL21DE3 cells were incubated for 10 minutes on ice, transferred into ice-cold electroporation cuvettes and shocked using the electroporator BioRad Micropulser (program: bacteria Ec2). After electroporation the cells were revitalized in 1 ml LB medium for 30 minutes at 37°C and 600 rpm. Then 100 µl of the cells and 100 µl of the 1:10 diluted cells (in LB media) were plated on LB agar plates containing the required antibiotics (100 µg/ml ampicillin or 40 µg/ml kanamycin). The plates were incubated over night at 37°C.

4.1.11 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The investigated protein solutions were separated by SDS-PAGE. These gels consisted of a resolving and a stacking gel, which were poured and polymerized successively. The volumes shown in table 12 are sufficient for producing 2 polyacrylamide gels. The Running buffer for these gels is listed in part 3.6.1.

Table 12: Composition of the polyacrylamide gel

Resolving gel (12 %)		**Stacking gel (4%)**	
H_2O	5.6 ml	H_2O	6 ml
30 % Acrylamide /Bis Solution (Bio-Rad Laboratories Inc)	6.4 ml	30 % Acrylamide /Bis Solution (Bio-Rad Laboratories Inc)	1.5 ml
1.5 M Tris-HCl 0.4 % SDS pH 8.8	4 ml	0.5 M Tris-HCl 0.4 % SDS pH 6.8	2.5 ml
10 % Ammonium persulfate (APS)	50 µL	10 % Ammonium persulfate (APS)	20 µL
Tetramethylethylenediamine (TEMED)	20 µL	Tetramethylethylenediamine (TEMED)	15 µL
		2 % Bromophenol blue	10 µL

Additionally NuPAGE® Novex® 4-12% Bis-Tris Gels (Life Technologies) were used to analyse the proteins. For these gels the 1x MES buffer was used (see 3.6.1 SDS-PAGE).

7.5 µL of the elution fraction, resuspended pellet or supernatant after sonication was mixed with 3 µL of 4x NuPAGE® LDS Sample Buffer and 0.5 µL of 1M DTT. The whole mixture was incubated at 95 °C for 10 min, centrifuged briefly and loaded onto the gel. To determine the size of the protein bands the PageRuler™ Prestained Protein Ladder (Thermo Scientific) was loaded onto the gel (figure 12).

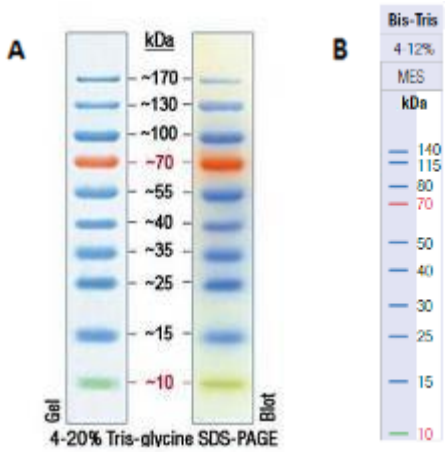

***Figure 12: Used protein standards**. (A) PageRuler™ Prestained Protein Ladder (Thermo Scientific) for 4-20% Tris-glycine SDS-PAGE. (B) PageRuler™ Prestained Protein Ladder (Thermo Scientific) for 4-12 % Bis-Tris SDS-PAGE and MES buffer.*

The electrophoresis was performed at 110 volt for 90 minutes using the SE 250 Mighty Small II electrophoresis unit and the PowerPac™ Basic Power Supply. After electrophoresis the gels were stained for 30 minutes in Coomassie Brilliant Blue staining solution and destained using 10% acetic acid.

4.1.12 Western blot

To determine, if the expression of the His_6-tagged proteins and the cell disruption was successful, a western blot analysis was performed. Samples of the cell pellet and the supernatant after sonication (insoluble and soluble fraction) were separated by SDS-PAGE. The gel was blotted onto a nitrocellulose membrane (Roth) for 1 hour. The membrane was blocked with 2.5 % BSA (bovine serum albumin, Carl Roth) in 1x TBS buffer for 1 hour. After blocking, the membrane was incubated with the primary antibody (Monoclonal Anti-polyHistidine, clone HIS-1, mouse IgG2a isotype, 1:3000 1% BSA in 1x TBS buffer, Sigma Aldrich H1029) over night at room temperature. Afterwards the membrane was washed 3 times for 5 minutes with 1x TBS buffer. The membrane was incubated with the secondary antibody (goat anti-mouse IgG-AP polyclonal, 1:5000 1% BSA in 1x TBS buffer, Santa Cruz Biotechnology sc-2008) for 1 hour at room temperature. The membrane was washed 3 times for 5 minutes with 1x TBS buffer. The His_6-tagged proteins were detected with the BCIP/NBT Kit (Life technology).

4.1.13 Cloning of DNA fragments

To incorporate DNA sequences of interest into a vector, the DNA sequence and the desired vector were treated with endonucleases as described in 4.1.7. To avoid self-ligation of the vector backbone, a dephosphorylation of the vector ends was performed. Therefore, 1 µl thermosensitive alkaline phosphatase (FastAP, 1 U/µl) and 3.5 µl 10x FastAP buffer were added to 30 µl linearized and purified vector DNA. This reaction mixture was incubated for 30 minutes at 37°C. Afterwards the phosphatase was inactivated at 65°C for 15 minutes. Finally the resulting DNA fragments were subject to a ligation reaction as described in 4.1.8. The generated plasmids were transformed into *E. coli* XL1-Blue (see 4.1.10) and subject to plasmid isolation as described in 4.1.2.

4.2 Construction of the pMS470nHis_ssb expression vector

The vector pMS470_ssb (generated during the project laboratory, see project laboratory protocol "Recombination experiments with linear DNA in *Lactobacillus plantarum*" for details) was double digested with *Nde*I and *Hind*III, to isolate the *ssb* fragment. This fragment was cloned into the vector pMS470nHis using *Nde*I and *Hind*III to generate the pMS470nHis_ssb plasmid (see figure 13). The vector pMS470nHis is designed to N-terminally fuse a gene of interest to a His_6-tag using the restriction enzymes *Nde*I and *Hind*III. A start codon inside the *Nco*I restriction site ensures the expression of a His_6- tagged protein (figure 14).

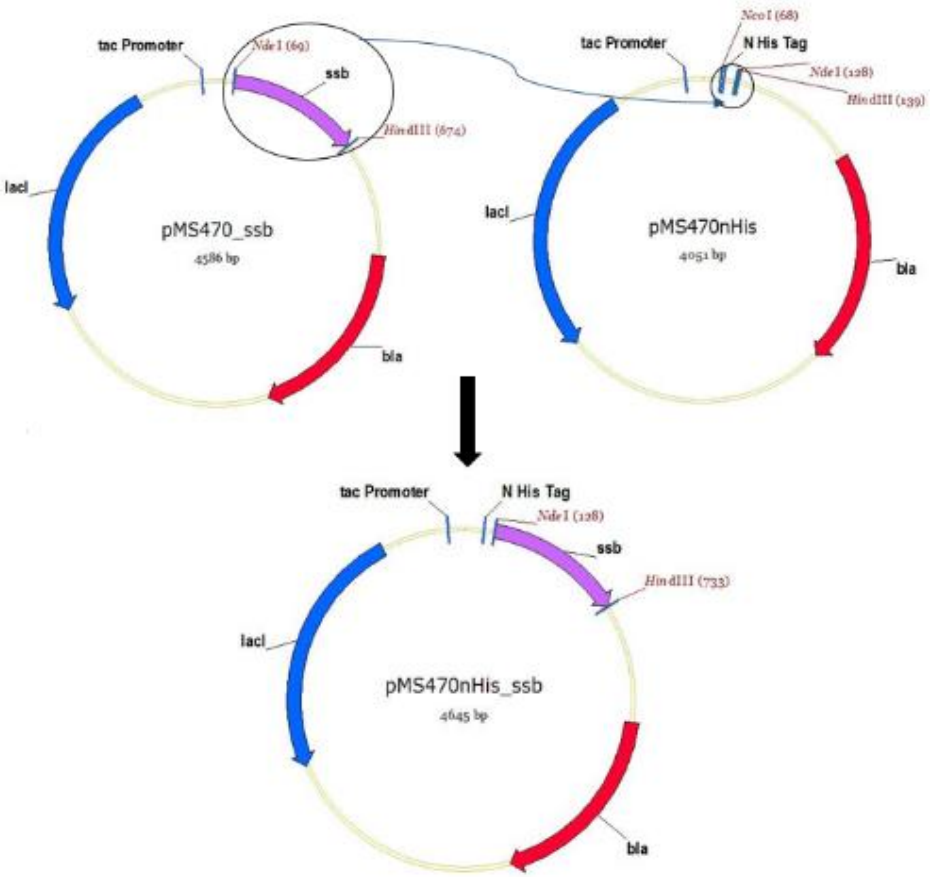

Figure 13: Illustration of the ssb fragment insertion into the pMS470nHis vector. *The positon of the resistance gene (bla), the repressor sequence (lacI), the promoter (tac) and the target gene (ssb) are shown. Additionally the restriction enzyme cleavage sites (NdeI and HindIII) for the cloning procedure are indicated.*

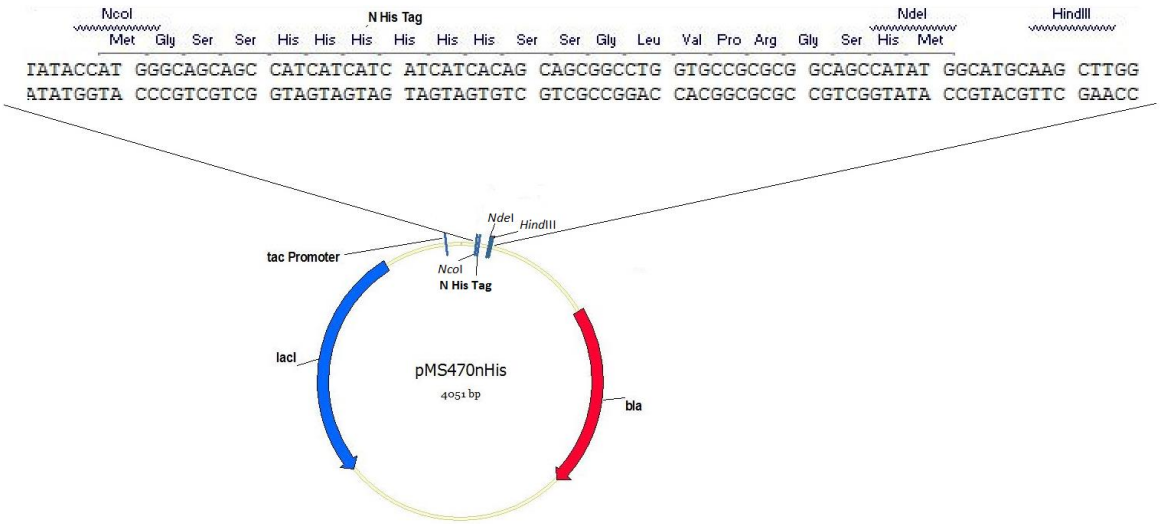

Figure 14: Sequence of the His_6 tag in the pMS470 vector. *The nucleotide sequence of the region between the NcoI and the HindIII restriction sites are shown. The Met inside the NcoI restriction site is the start codon of the His_6-tag. NdeI and HindIII are the restriction sites used to insert the gene of interest. The positon of the resistance gene (bla), the repressor sequence (lacI) and the promoter (tac) are shown.*

4.3 Construction of the pMS470nHis_recA expression vector

The plasmid pMS470_linker as well as the fragment of the *recA* gene were generated during the project laboratory (see project laboratory protocol "Recombination experiments with linear DNA in *Lactobacillus plantarum*" for details). The *recA* fragment was cloned into the pMS470_linker by double digestion with *Bgl*II and *Hind*III to generate the plasmid pMS470_linker_recA (figure 15). In the next step the *recA* gene was amplified by Standard PCR using the plasmid pMS470_linker_recA and the primers 166 and 137 (see table 3 primer list for details) to introduce an *Nde*I restriction site at the N-terminal end of the *recA* fragment. The PCR product was purified by agarose gel electrophoresis followed by gel slice purification using the Wizard® SV Gel and PCR Clean-Up System Kit (Promega) and cloned into the pJET1.2 vector (as described in 4.1.6) to generate the plasmid pJET_recA (figure 15). Because of the second *Nde*I restriction site inside the *recA* sequence, it was isolated from the plasmid pJET_recA by partial digestion with *Hind*III and *Nde*I for 30 minutes at 37°C with 50% of the recommended *Nde*I concentration. The isolated *recA* fragment was cloned into the vector pMS470nHis which was double digested with *Hind*III and *Nde*I to generate the plasmid pMS470nHis_recA (see figure 16).

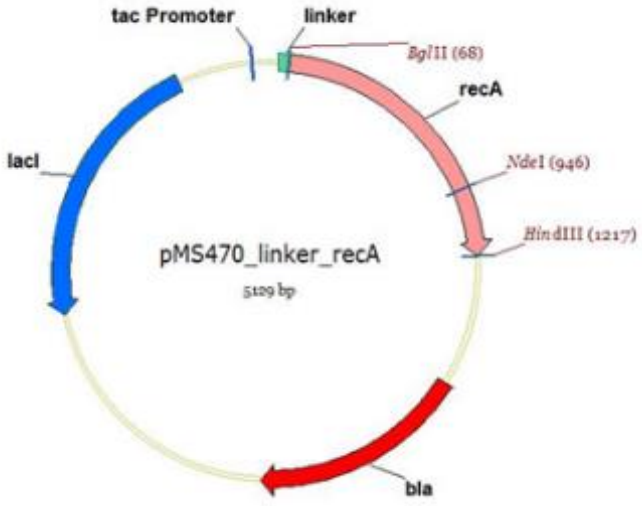

Figure 15: Plasmid map of the expression plasmid pMS470_linker_recA. *The positon of the resistance gene (bla), the repressor sequence (lacI), the promoter (tac) and the target gene (recA) is shown. Additionally the restriction enzyme cleavage sites used for the cloning procedure are indicated.*

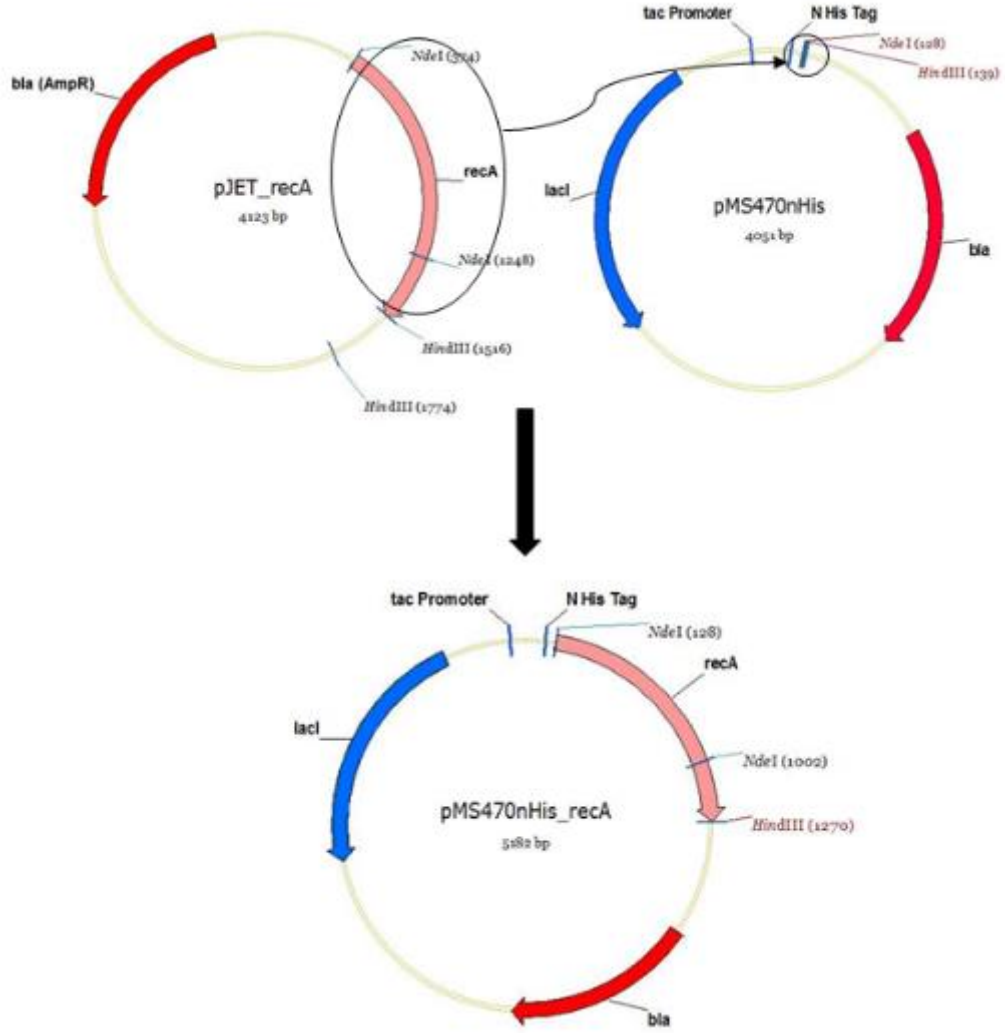

Figure 16: Illustration of the recA fragment insertion into the pMS470nHis vector. *The positon of the resistance gene (bla), the repressor sequence (lacI), the promoter (tac) and the target gene (recA) are shown. Additionally the restriction enzyme cleavage sites (NdeI and HindIII) for the cloning procedure are indicated.*

4.4 Heterologous expression of the N-terminal His_6-tagged *L. plantarum* protein SSB and RecA in *E.coli*

For the heterologous expression of the *L. plantarum* protein SSB, the strains *E. coli* BL21DE3 [pMS470nHis_ssb] and *E. coli* BL21DE3 [pET28b_ssb] (plasmid maps in figure 17) were used. *E. coli* BL21DE3 [pET28b_ssb] was generated during the project laboratory (see project laboratory protocol "Recombination experiments with linear DNA in *Lactobacillus plantarum*" for details). For the heterologous expression of the *L. plantarum* protein RecA, the strain *E. coli* BL21DE3 [pMS470nHis_recA] (plasmid map in figure 18) was used.

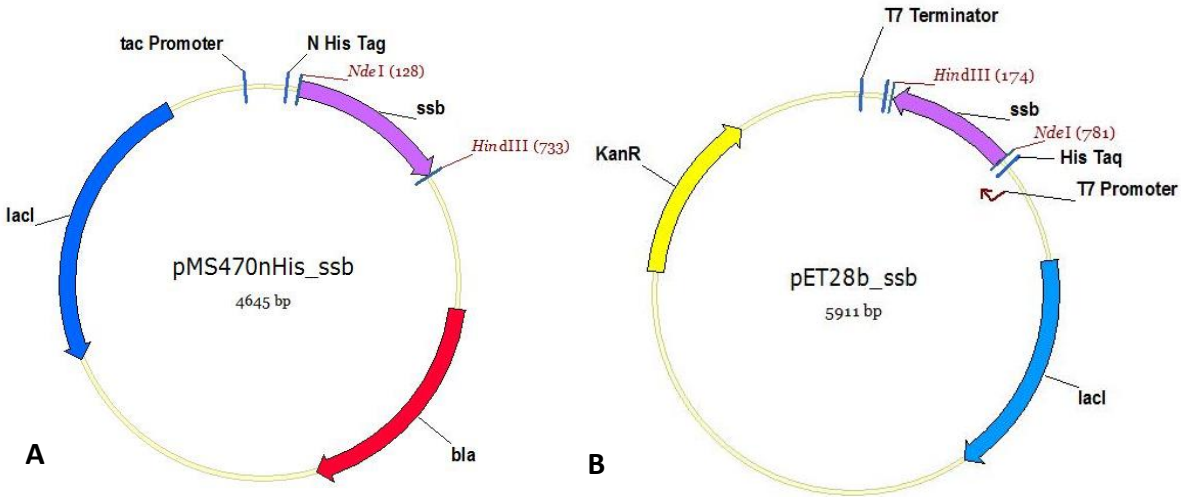

Figure 17: Plasmid map of the expression plasmid pMS470nHis_ssb (A) and pET28b_ssb (B). *The positon of the resistance gene (bla or KanR), the repressor sequence (lacI), the promoter (tac or T7) and the target gene (ssb) are shown. Additionally the position of the His_6-tag and restriction enzyme cleavage sites used for the cloning procedure are indicated.*

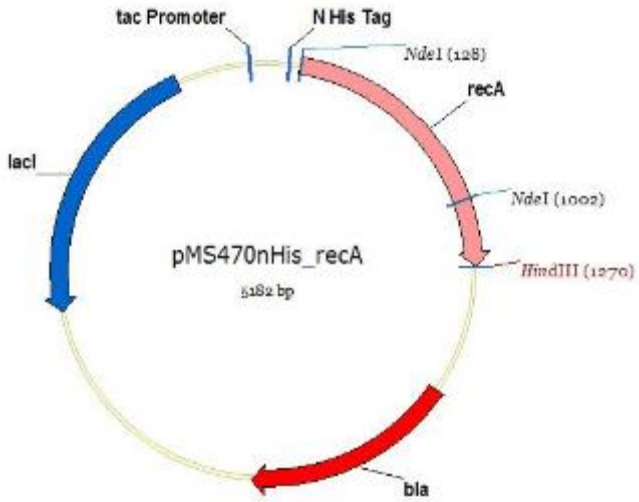

Figure 18: Plasmid map of the expression plasmid pMS470nHis_recA. *The positon of the resistance gene (bla), the repressor sequence (lacI), the promoter (tac) and the target gene (recA) is shown. Additionally the position of the His_6-tag and restriction enzyme cleavage sites used for the cloning procedure are indicated.*

The expression strains were precultured in 5 ml LB-Amp or LB-Kan at 37°C and 120 rpm over night. The 100 ml LB-Amp main culture were inoculated with the overnight culture to an OD_{600} of 0.1. The main cultures were incubated at 37 °C and 120 rpm to an OD_{600} of 0.6, afterwards the expression of the proteins were induced by addition of 100 µl 100 mM IPTG (final concentration 0.1 mM). The induced main cultures were incubated at 28°C and 120 rpm over night. After 20 hours of induction, the cells were harvested by centrifugation at 3220 x g and 4°C (Eppendorf Centrifuge 5810R) for 30 minutes. The cell pellets were stored at -20 °C.

4.5 Protein purification of the His_6-tagged proteins

The frozen cell pellets were resuspended in 30 ml Lysis Buffer and were disrupted by sonication (3 times for 5 minutes, Branson Sonifier 250 Duty Cycle % 80; Output Control 8). After the cell disruption the suspension was centrifuged at 3220 x g and 4°C (Eppendorf Centrifuge 5810R). The supernatant of the proteins were then sterile filtered (Sarstedt Filtropur S0.2, PES-Membran, 0.2 µm) and cooled on ice. The heterologously expressed His_6-tagged proteins were purified using a Ni-NTA sepharose column (GE Healthcare 17-

5318). The column was equilibrated two times with 2 CV (column volume) Lysis Buffer. Afterwards the cell lysates were loaded onto columns and the flow-through was collected. The columns were then washed 2 times with 1 CV 30 mM imidazole Wash Buffer, followed by 2 times 1 CV 40 mM imidazole Wash Buffer to remove unbound proteins and the flow-through was collected. The His_6-tagged proteins were eluted 6 times with 0.5 ml Elution Buffer (10 minutes incubation time for each step). The elution fractions containing the protein were pooled and rebuffered in the corresponding Storage Buffer by using the PD-10 desalting column (GE Healthcare, 17-0851-01) gravity protocol. The proteins were stored at -80°C until they were used for the further experiments.

4.5.1 PD-10 desalting column gravity protocol

The PD-10 column was equilibrated with 25 ml of the corresponding Storage Buffer described in section 3.6.3. The elution fractions (max 2.5 ml) were added to the column. The proteins were eluted with 3.5 ml of the corresponding Storage Buffer and collected in a fresh tube.

4.5.2 Vivaspin®4 centrifugal concentration protocol

The rebuffered protein solutions were loaded into the Vivaspin®4 column (PES membrane, 10000 MWCO, Sartorius stedim biotech) and were centrifuged at 4°C and 3220 x g until a final volume of 500 µl was achieved.

The protein concentrations were measured spectrophotometrically at a wavelength of 280 nm using NanoDrop 2000c (Thermo Scientific). The extinction coefficient for SSBnHis is 14440 $M^{-1}*cm^{-1}$ and for RecAnHis 20400 $M^{-1}*cm^{-1}$ as calculated by the ExPASy ProtParam tool using the SSBnHis and RecAnHis protein sequence indicated in the appendix.

4.6 Generation of linear ssDNA

For the generation of linear ssDNA asymmetric PCR was performed using the plasmid pMS470_5´3´cbh_CAT as template (figure 19). This plasmid has been generated during the project laboratory (see project laboratory protocol "Recombination experiments with linear DNA in *Lactobacillus plantarum*" for details) and contains the homologous region of the *cbh* gene directly isolated from *L. plantarum* CD033 (see project laboratory protocol "Recombination experiments with linear DNA in *Lactobacillus plantarum*" for details). The vector pMS470 is usually used as an expression vector which enables the induction of protein expression upon addition of IPTG (isopropyl β-D-1-thiogalactopyranoside). However in this case the vector simply acts as a vehicle to carry the linear integration cassette.

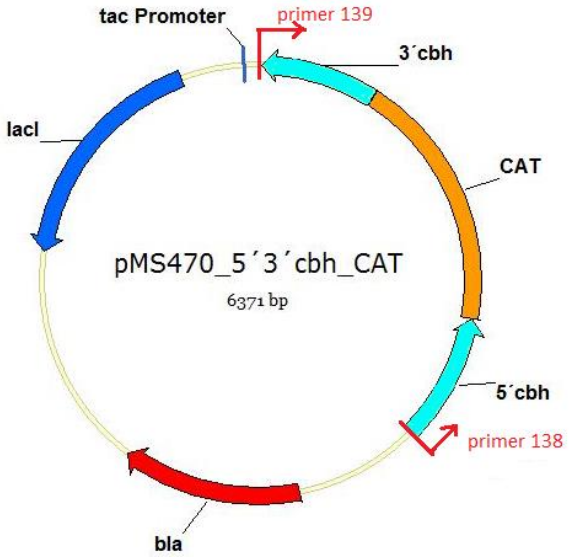

Figure 19: Plasmid map of the pMS470_5´3´cbh_CAT plasmid. *Shown are the homologous regions of the cbh gene and the chloramphenicol acetyl transferase (CAT) cassette. The position of the used primers for the asymmetric PCR are marked in red.*

Asymmetric PCR describes a method that preferentially amplifies one strand of the target DNA because of unequal addition of either the forward or the reverse primers.
Therefore either the forward or the reverse primer was used in 100-fold excess for the PCR with the plasmid pMS470_5´3´cbh_CAT as template. The used PCR programs and reaction mixtures are summarized in table 13 to 16. The resulting DNA of the PCR using program A was subsequently used for the integration experiments in *L. plantarum* and the resulting DNA of the PCR using program B was subsequently used for the Gel shift assays.

Table 13: Thermo cycler program A

98°C	3 min	
98°C	30 sec	25 cycles
53°C	30 sec	
72°C	3 min	
72°C	5 min	
4°C	∞	

Table 14: Reaction mix for thermo cycler program A:

I) The mix for the forward primer in excess, II) The mix for the reverse primer in excess is shown.

I)	Component [µl]
2	Template DNA*
5	Primer 138 (10 µM)
1	Primer 139 (0.5 µM)
10	5x HF Buffer
1	10 mM dNTPs
30.8	Sterile H_2O
0.2	Phusion Polymerase (2 U/µl)

II)	Component [µl]
2	Template DNA*
1	Primer 138 (0.5 µM)
5	Primer 139 (10 µM)
10	5x HF Buffer
1	10 mM dNTPs
30.8	Sterile H_2O
0.2	Phusion Polymerase (2 U/µl)

*concentration of the template (cccDNA of pMS470_5´3´cbh_CAT) was not determined

Table 15: Thermo cycler program B

98°C	30 sec	
98°C	10 sec	35 cycles
58°C	30 sec	
72°C	90 sec	
72°C	5 min	
4°C	∞	

Table 16: Reaction mix for thermo cycler program B:

I) The mix for the forward primer in excess, II) The mix for the reverse primer in excess is shown.

I)	Component [µl]
1	Template DNA (16.5 ng)
5	Primer 138 (10 µM)
1	Primer 139 (0.5 µM)
10	5x HF Buffer
1	10 mM dNTPs
31.5	Sterile H_2O
0.5	Phusion Polymerase (2 U/µl)

II)	Component [µl]
1	Template DNA (16.5 ng)
1	Primer 138 (0.5 µM)
5	Primer 139 (10 µM)
10	5x HF Buffer
1	10 mM dNTPs
31.5	Sterile H_2O
0.5	Phusion Polymerase (2 U/µl)

*concentration of the template (cccDNA of pMS470_5´3´cbh_CAT) was determined by NanoDrop 2000c

The resulting linear ssDNA of the short integration cassette, 5´3´cbh_CAT, which contains the homologous region of the *cbh* gene and the *chloramphenicol acetyl transferase* (*CAT*) cassette (figure 20), was used for the preliminary DNA-protein loading (Gel shift assay) and integration experiments in *L. plantarum.*

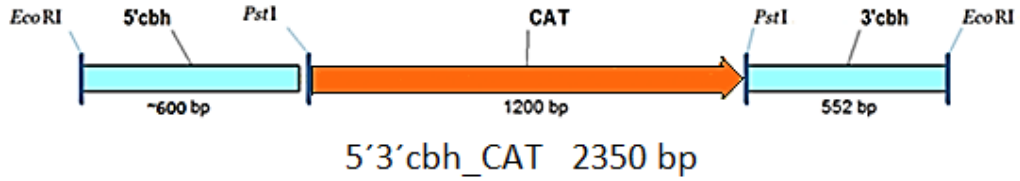

Figure 20: Short linear integration cassette. *Shown are the homologous regions of the cbh gene and the chloramphenicol acetyl transferase (CAT) cassette.*

4.6.1 Ethanol precipitation of the linear ssDNA

The ethanol precipitation was used to increase the concentration of the linear ssDNA acquired from the asymmetric PCR using either the forward or the reverse primer in 100-fold excess. One volume of the DNA, 0.1 volume 3 M sodium acetate and 2.5 volume of 96 % ethanol (-20°C) were mixed and incubated at -20°C over night. Afterwards the mixture was centrifuged at 16100 x g for 30 minutes at 4°C. After two washing steps using 70 % ice-cold ethanol, the DNA was dried at 37°C. The DNA pellet was then dissolved in 20-30 µl sterile nuclease-free water. DNA concentration was measured at a wavelength of 260 nm by NanoDrop 2000c (Thermo Scientific).

4.7 Construction of the Flp/*frt* based integration cassette

The Flp/*frt* based cassette was used for the integration experiments into the genome of the *L. plantarum* strains CD033 and 3NSH, to generate a *L. plantarum* strain which contains a cellulase in his genome. This cassette contains the homologous regions of the conjugated bile acid hydrolase gene (*cbh)*, the cellulase gene (*celAocES,* modified endocellulase from *Sorangium cellulosum*) under the control of a promoter (*Lactobacillus buchneri* CD034 elongation factor P promoter, *efp* promoter), a terminator sequence (*Lactobacillus casei* L-lactat dehydrogenase terminator, T_{ldh}), the *CAT (chloramphenicol acetyl transferase)* cassette and the *frt* sites for the Flp/*frt* recombination system (see figure 21).

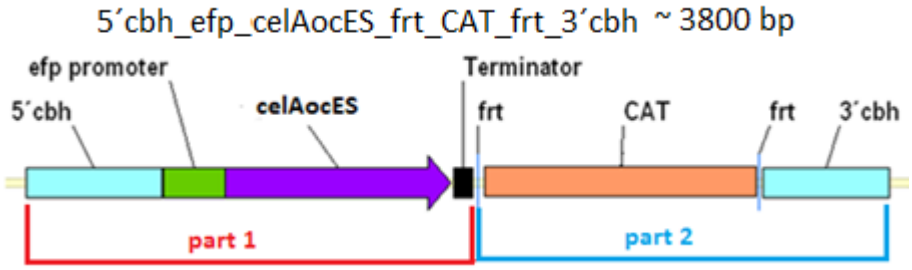

Figure 21: Schematic representation of the linear Flp/frt based integration cassette*. The cassette 5´cbh_efp_celAocES_frt_CAT_frt_3´cbh contains the homologous regions of the cbh gene, the chloramphenicol acetyl transferase (CAT) cassette, the efp promoter, the modified cellulase gene from Sorangium cellulosum (celAocES), a terminator sequence (L. casei L-lactat dehydrogenase terminator, T_{ldh}) and the frt sites for the Flp/frt recombination system. The 5´cbh_efp_celAocES moiety (part1) and the frt_CAT_frt_3´cbh moiety (part2) have been cloned separately and finally combined to give the full construct.*

Due to its size and complexity the Flp/*frt* based integration cassette has been cloned as two separate parts (part 1 and 2 indicated in figure 21) which finally have been joint together to give the full length integration cassette 5´cbh_efp_celAocES_frt_CAT_frt_3´cbh as shown in figure 21. The general methods used for the construction of the Flp/*frt* based integration cassette were performed as described in 4.1.

Cloning of the 5´cbh_efp_celAocES construct (part 1)

Because cloning of small gene fragments like a terminator sequence can be a difficult task, all fragments required to generate the 5´cbh_efp_celAocES construct were cloned into the plasmid pINT_terminator.

Therefore the first step was to exchange the *5´cbh* homologous region of the plasmid pINT_terminator, which is about 100 bp larger than the previously used *5´cbh* of the short integration cassette (5´3´cbh_CAT), with the *5´cbh* of the pMS470_5´3´cbh_CAT plasmid using the restriction enzymes *Eco*RI and *Pst*I. The generated plasmid was renamed to pINT_term_5´cbhnew (figure 22).

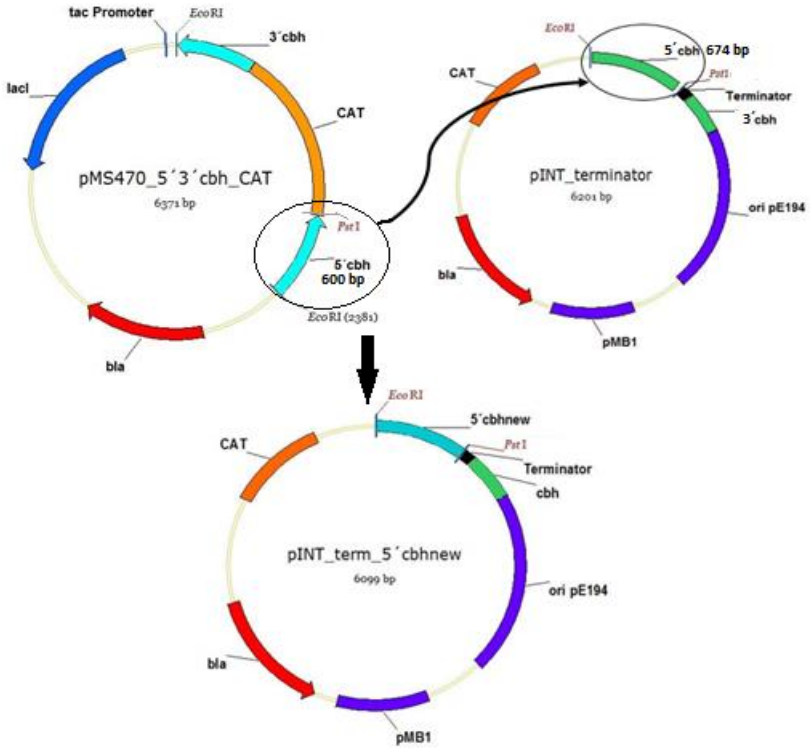

Figure 22: Scheme of the 5´cbh exchange*. The 5´cbh of the pINT_terminator was replaced by the 5´cbh of the plasmid pMS470_5´3´cbh_CAT after double digestion with EcoRI and PstI. The newly generated pINT_term_5´cbhnew contains a 5´cbh that is about 100 bp shorter than the 5´cbh of the original pINT_terminator. Additionally the positions of antibiotic resistance genes, ampicillin resistance (bla) and chloramphenicol acetyl transferase (CAT), the repressor sequence (lacI), the promoter (tac), the homologous regions cbh, the origin pMB1 for replication in E.coli and the unstable origin pE194 for replication in L. plantarum are shown.*

Furthermore the plasmid pINT_efp_celAocES which already contains the *celAocES* gene under the control of the *efp* promoter was used for the generation of the 5´cbh_efp_celAocES construct. The *efp* promoter in this plasmid, which naturally exists as a promotor for the elongation factor P in *L. buchneri* that is involved in the ribosomal protein synthesis, contains remains of the gene encoding the ribosomal protein L27 (from *L. buchneri*) and remains of the *CAT* sequence, which could be artefacts from the initial isolation and cloning of the *efp* promoter. In accordance with the CD laboratory for Genetically Engineered Lactic Acid Bacteria of the BOKU Vienna, the *efp* promoter was modified. Therefore the *efp* promoter of the plasmid pINT_efp_celAocES was amended by Standard PCR using the primers 189 and 188 (for efpshort1) as well as 190 and 188 (for efpshort2, see table 4 primer list for details). Two different variants of the *efp* promoter were generated, efpshort1 (remains of the gene encoding the ribosomal protein L27 were eliminated) and efpshort2 (remains of the gene encoding the ribosomal protein L27 and *CAT* sequence were eliminated) as shown in figure 23. The respective PCR products (efpshort1 and efpshort2) were cloned into the plasmid pINT_efp_celAocES by double digestion with *Sac*I and *Bgl*II. This resulted in the plasmids with modified *efp* promoter's pINT_efpshort1_celAocES and pINT_efpshort2_celAocES (figure 22). After plasmid isolation the plasmids pINT_term_5´cbhnew, pINT_efp_celAocES and the versions with the modified *efp* promoter (pINT_efpshort1_celAocES and pINT_efpshort2_celAocES), were double digested with *Pst*I and *Sac*I to clone the efp_celAocES or the respective versions with the modified promoters into the pINT_term_5´cbhnew vector. The generated plasmids were named pINT_term_5´cbh_efp_celAocES, pINT_term_5´cbh_efpshort1_celAocES and pINT_term_5´cbh_efpshort2_celAocES (figure 23).

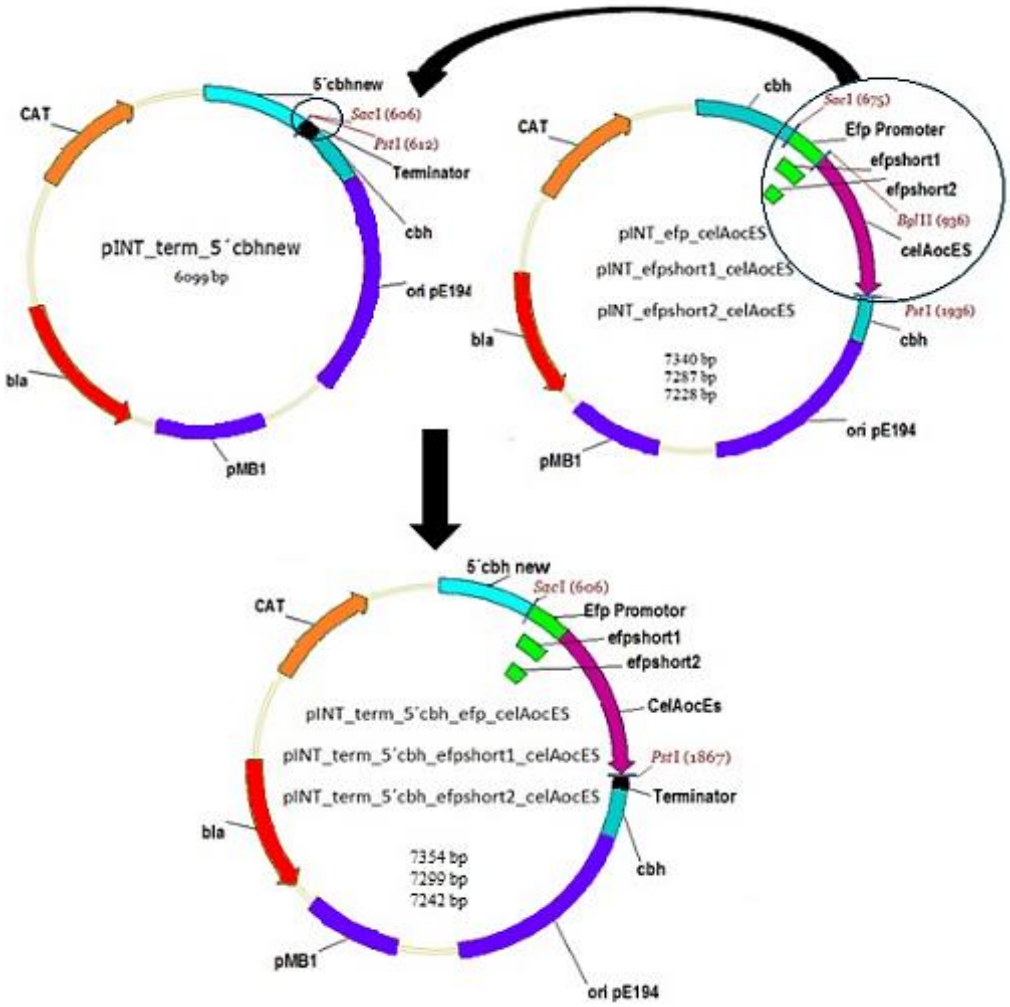

Figure 23: Illustration of the efp_celAocES fragments insertion. *The efp_celAocES or the respective versions with the shortened promoter fragments were cloned into the pINT_term_5´cbhnew vector by double digestion with PstI and SacI. The newly generated pINT_term_5´cbh_efp_celAocES, pINT_term_5´cbh_efpshort1_celAocES and pINT_term_5´cbh_efpshort2_celAocES plasmids contain the original efp promoter fragment or the shortened versions and the endocglucanase gene celAocES. Additionally the positions of antibiotic resistance genes, ampicillin resistance (bla) and chloramphenicol acetyl transferase (CAT), the homologous regions cbh, the origin pMB1 for replication in E.coli and the unstable origin pE194 for replication in L. plantarum are shown.*

Cloning of the frt_CAT_frt_3´cbh construct (part 2)

In order to generate the frt_CAT_frt_3´cbh moiety of the Flp/*frt* based integration cassette the *frt*-sites had to be attached to the *CAT* and *3´cbh* sequences which were joint together. To this purpose the CAT and 3´cbh sequences of the plasmid pMS470_5´3´cbh_CAT were used as template for a Standard PCR using primers that included the 34 bp *frt*-sites (figure 24, primer 180-183 see table 4).

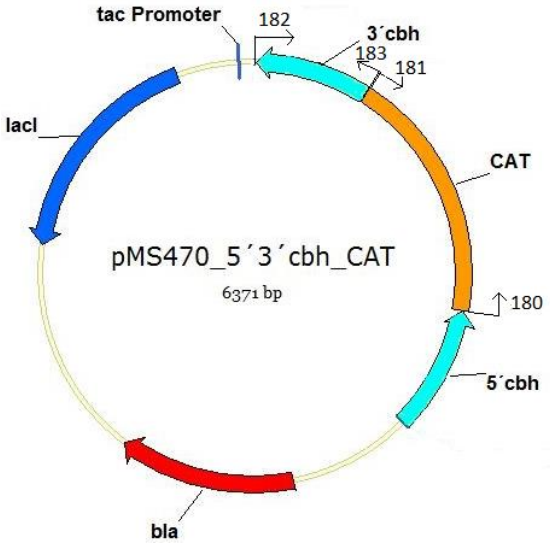

Figure 24: Plasmid map of the pMS470_5´3´cbh_CAT plasmid. *Shown are the homologous regions of the cbh gene and the chloramphenicol acetyl transferase (CAT) cassette. Additionally, the position of the used primers for the amplification of the frt_CAT and frt_3´cbh fragment are shown.*

The PCR resulted in the fragments frt_CAT and the frt_3´cbh which were cloned into the pJET1.2 blunt using the CloneJET™ PCR Cloning Kit (Thermo scientific) to generate the plasmids pJET_frt_CAT and pJET_frt_3´cbh (figure 25). The pJET1.2 blunt vector was used as a vehicle for the generated fragments to enable the further cloning steps.

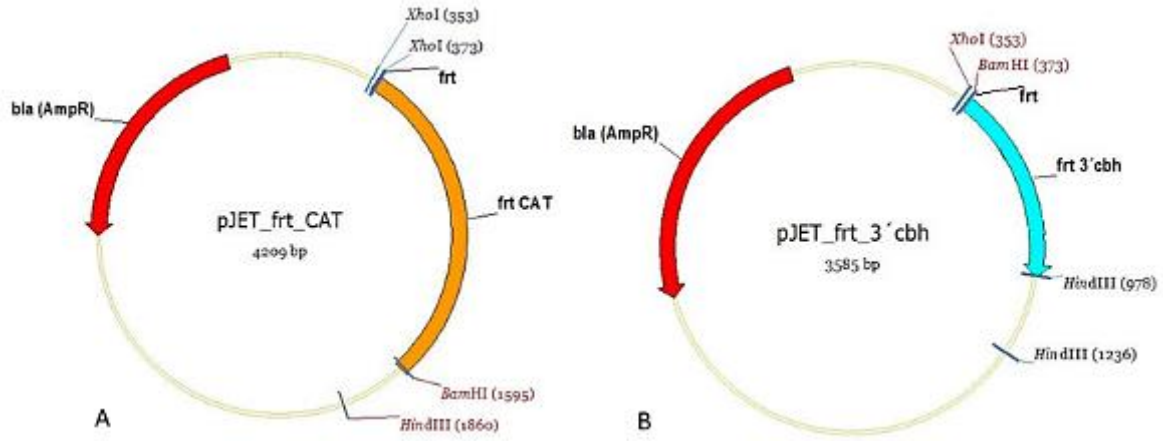

Figure 25: Plasmid maps of pJET_frt_CAT (A) and pJET_frt_3´cbh (B). *The positon of the resistance gene (bla) and the fragments frt_CAT (A), frt_3´cbh (B) are shown. Additionally the position of the restriction enzyme cleavage sites used for the following cloning procedure are indicated.*

Next, the pJET_frt_CAT was digested with *Bam*HI and *Hind*III and the frt_3´cbh fragment was excised from the plasmid pJET_frt_3´cbh by double digestion with *Bam*HI and *Hind*III followed by agarose gel purification. Afterwards the frt_3´cbh fragment was ligated with the linearized pJET_frt_CAT to generate the plasmid pJET_fcf3c (figure 26).

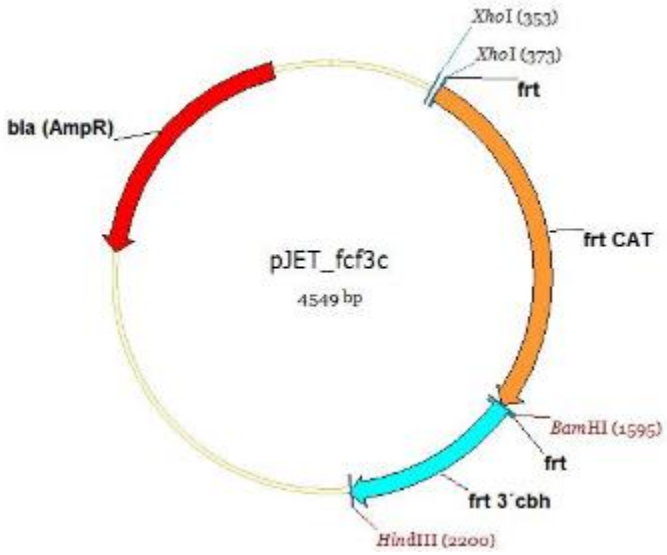

Figure 26: Plasmid map of pJET_fcf3c. *The positon of the resistance gene (bla) and the final fragment (frt_CAT_frt_3´cbh) are shown. Additionally the position of the used restriction enzyme cleavage sites are indicated in red.*

Linkage of the 5´cbh_efp_celAocES constructs and the frt_CAT_frt_3´cbh construct to generate the 5´cbh_efp_celAocES_frt_CAT_frt_3´cbh integration cassettes.

First of all the 5´cbh_efp_celAocES constructs (with the different promoter modifications) were isolated from the respective plasmids (pINT_term_5´cbh_efp_celAocES, pINT_term_5´cbh_efpshort1_celAocES and pINT_term_5´cbh_efpshort2_celAocES) by double digestion with *Eco*RI and *Xho*I followed by agarose gel purification. Simultaneously the frt_CAT_frt_3´cbh construct was also isolated by double digestion with *Xho*I and *Hind*III followed by agarose gel purification. Afterwards each of the 5´cbh_efp_celAocES fragment versions were ligated to the frt_CAT_frt_3´cbh fragment using 50 ng of each fragment. Subsequently the ligated constructs were purified using the Wizard®SV Gel and PCR Clean-Up System (Promega) and amplified by Standard PCR using the primers 122 and 183 (see table 3).

The resulting 5´cbh_efp_celAocES_frt_CAT_frt_3´cbh fragment and the versions with the shortened promoter were purified by agarose gel electrophoresis and cloned into the pJET1.2 blunt vector. The pJET1.2 blunt vector was chosen because it enables fast and easy blunt end cloning which was required to successfully clone the blunt end 5´cbh_efp_celAocES_frt_CAT_frt_3´cbh fragment and the versions with the shortened promoter into a vector. The vectors containing the complete 5´cbh_efp_celAocES_frt_CAT_frt_3´cbh fragment as well as the versions with the shortened promoter were used for fragment isolation and long term storage.

A summary of the cloning strategy of the Flp/*frt* based integration cassette is shown in figure 27.

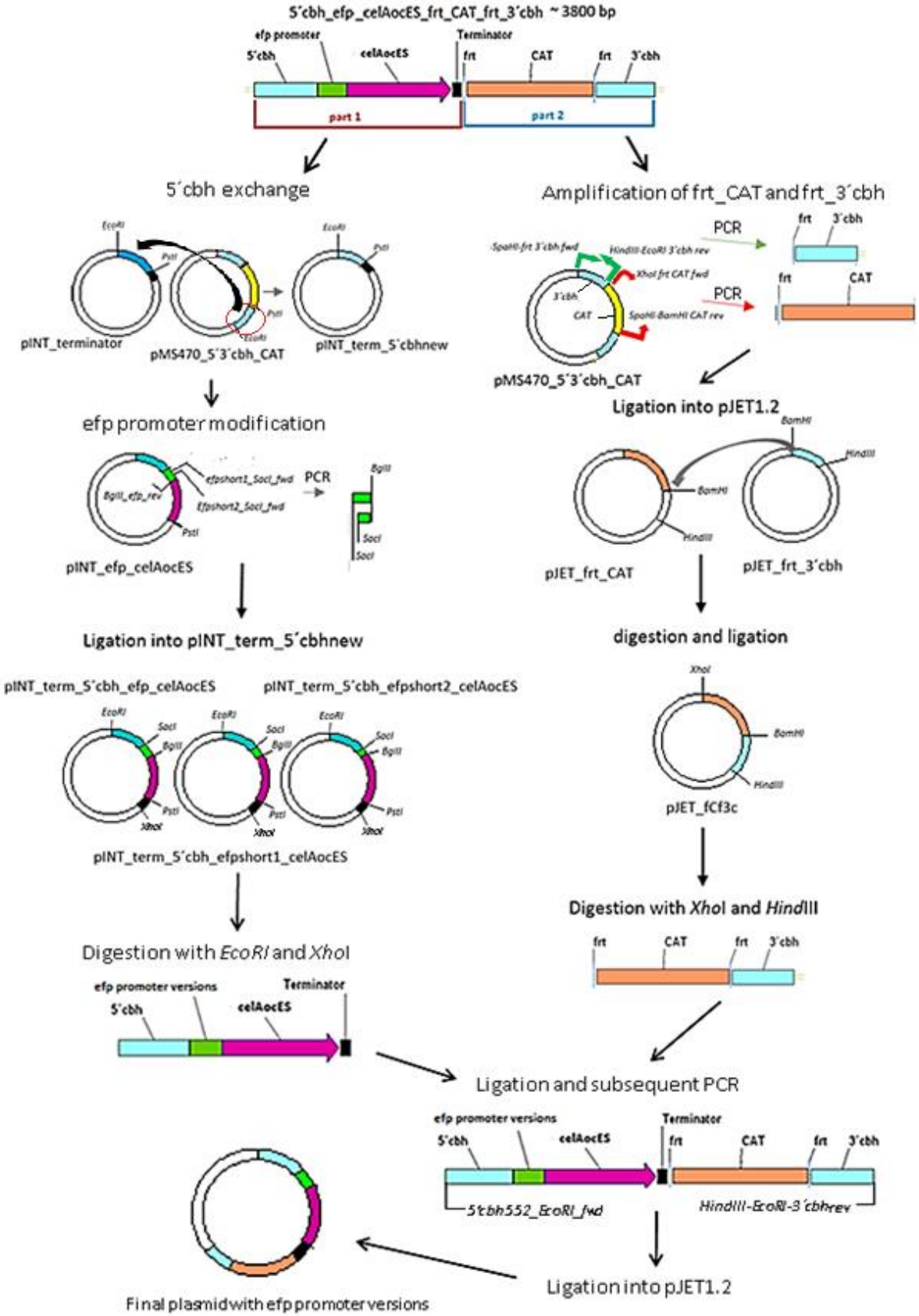

Figure 27: Schematic representation of the cloning strategy used to generate the Flp/frt based integration cassette.

4.8 Generation of linear dsDNA

For the generation of linear dsDNA Standard PCR (see 4.1.3) using the plasmids pMS470_5´3´cbh_CAT (figure 28), pJET_5´cbh_efp_celAocES_fcf3c (figure 30 A) and pJET_5´cbh_efpshort2_celAocES_fcf3c (figure 30 B) as templates and the primers 138 and 139 (see table 5 for details) was performed. The resulting dsDNA of the integration cassette 5´3´cbh_CAT, (figure 28) was used for DNA/protein loading experiments (Gel shift assay) and integration experiments in *L. plantarum.* The linear dsDNA of the 5´cbh_efp_celAoc_frt_CAT_frt_3´cbh cassette (figure 31) and 5´cbh_efpshort2_celAoc_frt_CAT_frt_3´cbh cassette (figure 32) were used for integration experiments in *L. plantarum* only.

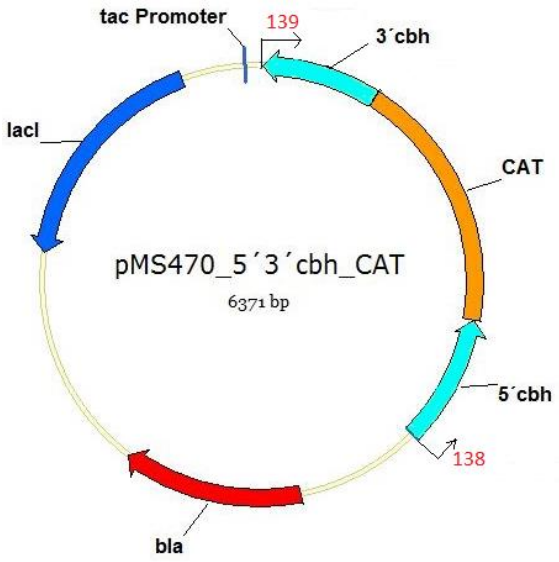

Figure 28: Plasmid map of the pMS470_5´3´cbh_CAT plasmid. *Shown are the homologous regions of the cbh gene and the chloramphenicol acetyl transferase (CAT) cassette. The position of the used primers for the asymmetric PCR are marked in red.*

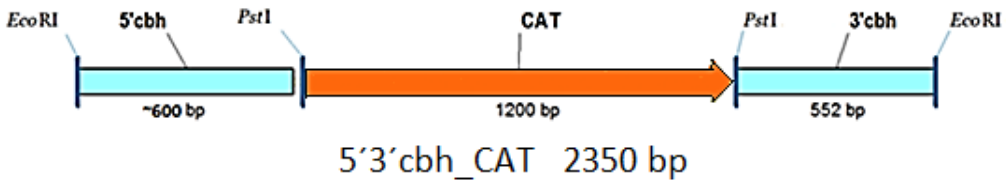

Figure 29: Short linear integration cassette. *Shown are the homologous regions of the cbh gene and the chloramphenicol acetyl transferase (CAT) cassette.*

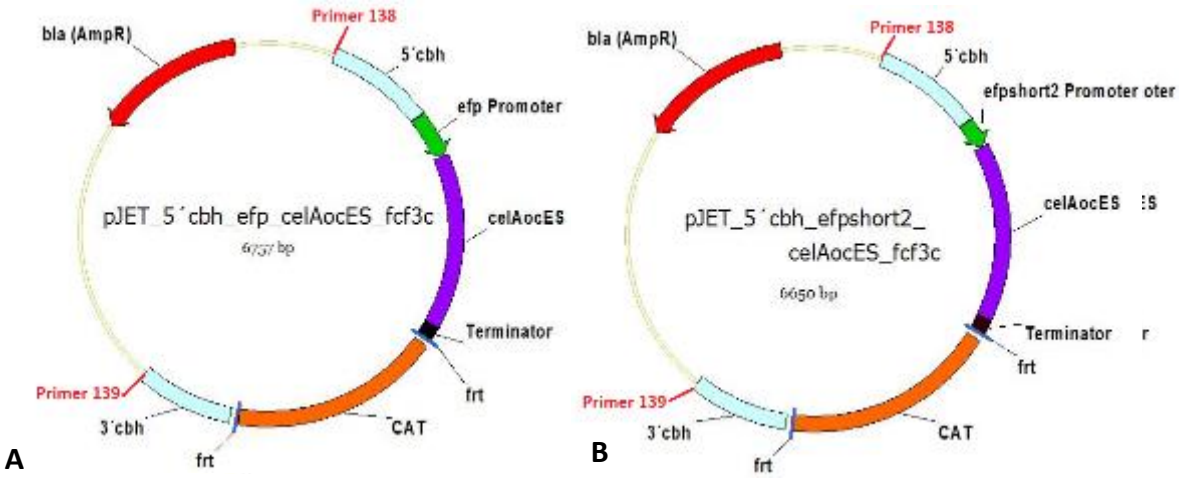

Figure 30: Plasmid maps of the pJET_5´cbh_efp_celAocES_fcf3c (A) and pJET_5´cbh_efpshort2_celAocES_fcf3c (B). *Shown are the homologous regions of the cbh gene, the efp promoter (A) and efpshort2 promoter (B), the endoglucanase gene (celAocES), the frt sites and the chloramphenicol acetyl transferase (CAT) cassette. The position of the used primers for the asymmetric PCR are marked in red.*

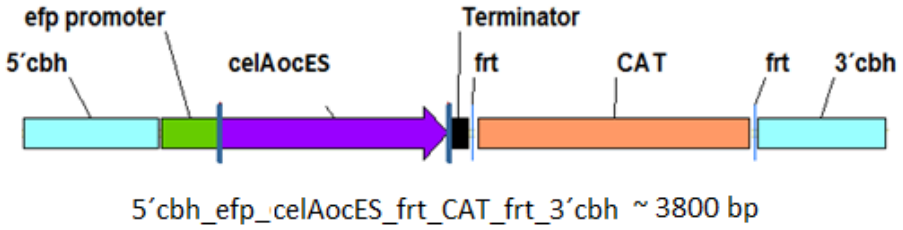

Figure 31: Scheme of the linear Flp/frt based integration cassette 5´cbh_efp_celAocES_fcf3c. *Shown are the homologous regions of the cbh gene, the efp promoter, the endoglucanase gene (celAocES), the terminator (T_{ldh}), frt sites and the chloramphenicol acetyl transferase (CAT) cassette.*

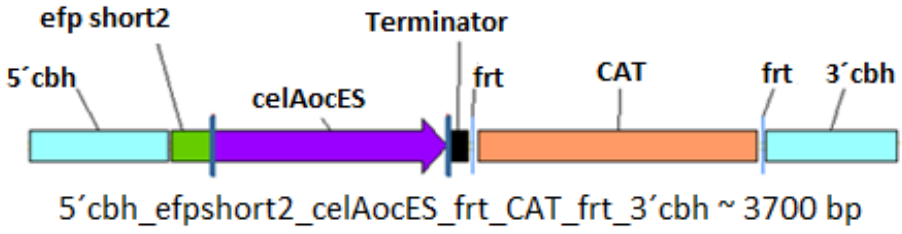

Figure 32: Scheme of the linear Flp/frt based integration cassette 5´cbh_efpshort2_celAocES_fcf3c. *Shown are the homologous regions of the cbh gene, the efpshort2 promoter, the endoglucanase gene (celAocES) the terminator (T_{ldh}), frt sites and the chloramphenicol acetyl transferase (CAT) cassette.*

Successful amplification of the integration cassette was determined by agarose gel electrophoresis, the PCR products were purified using the Wizard®SV Gel and PCR Clean-Up System (Promega) and the resulting DNA concentration was measured at a wavelength of 260 nm by NanoDrop 2000c (Thermo Scientific).

4.9 Gel shift assay

The gel shift or mobility shift assay is an affinity electrophoresis technique used to study DNA-protein or RNA-protein interactions. The principle behind this method relies on the fact that DNA-protein complexes migrate slower than DNA alone in a native polyacrylamide gel or agarose gel. This difference in electrophoretic separation of DNA-protein complexes can be visualized as a "shift" in migration of the labelled DNA band. The ssDNA and dsDNA was loaded with the proteins SSBnHis and RecAnHis to detect differences in the DNA migration capacity compared to DNA alone. Also the non DNA binding protein alcohol dehydrogenase (ADH isolated from *Ralstonia eutropha* H16 and kindly provided by Zalina Magomedova) was used as a negative control. The mixtures (summarized in tables 17-19) were incubated at 22°C for 45 minutes. Afterwards 5 µl Loading Dye (0.05 % bromophenol blue, 40 % sucrose dissolved in 1 ml) were added. The DNA-protein interaction compared to non-loaded DNA was investigated by agarose gel electrophoresis (0.85% agarose gel and 80 volt).

Table 17: Reaction mixture for the gel shift assay approach I. The putative ssDNA was generated by asymmetric PCR using only the forward or reverse primer and the plasmid pMS470_5´3´cbh_CAT as template.

Approach I	
4, 2 and 2.5 µl	ssDNAfor, ssDNArev and dsDNA(~20 ng)*
0,1,3 µl	SSBnHis (0, ~2, ~ 6 µg) ǂ
x µl	SSB Storage buffer
10 µl	Final volume

** 20 ng of ssDNAfor equals 2.72E-5 nmol; 20 ng of ssDNArev equals 2.73E-5 nmol; 20 ng dsDNA equals 1.36E-5 nmol*

ǂ 2 µg SSBnHis equals 0.0851 nmol; 6 µg SSBnHis equals 0.255 nmol

Table 18: Reaction mixture for the gel shift assay approach II. The putative ssDNA was generated by asymmetric PCR using only the forward or reverse primer and the plasmid pMS470_5´3´cbh_CAT as template

Approach II	
4, 2 and 2.5 µl	ssDNAfor, ssDNArev and dsDNA(~20 ng)*
0, 1, 3 µl	RecAnHis (0, ~2, ~ 6 µg) ǂ
0.2 µl	ATP (50 mM)
x µl	RecA assay buffer
10 µl	Final volume

** 20 ng of ssDNAfor equals 2.72E-5 nmol; 20 ng of ssDNArev equals 2.73E-5 nmol; 20 ng dsDNA equals 1.36E-5 nmol*

ǂ 2 µg RecAnHis equals 0.0460 nmol; 6 µg RecAnHis equals 0.138 nmol

Table 19: Reaction mixture for the gel shift assay approach III. The putative ssDNA was generated by asymmetric PCR using only the forward or reverse primer and the plasmid pMS470_5´3´cbh_CAT as template

Approach III	
4, 2 and 2.5 µl	ssDNAfor, ssDNArev and dsDNA(~20 ng)*
0, 1.5, 4.6 µl	ADHnHis (0, ~2, ~ 6 µg) ǂ
x µl	ADH buffer
10 µl	Final volume

** 20 ng of ssDNAfor equals 2.72E-5 nmol; 20 ng of ssDNArev equals 2.73E-5 nmol; 20 ng dsDNA equals 1.36E-5 nmol*

ǂ 2 µg ADHnHis equals 0.0741 nmol; 6 µg ADHnHis equals 0.222 nmol

4.10 DNA/protein loading and transformation into *L. plantarum*

In order to investigate the effect of SSBnHis and RecAnHis on the integration efficiency, empty ssDNA (0.5 and 5 µg), empty dsDNA (5 µg) and the approaches A-D summarized in the tables 20 and 21 were used for the transformation into *L. plantarum* CD033 and 3NSH. The protein and DNA were mixed and incubated for 1 hour at 22°C. Afterwards the separate approaches were subject to transformation into *L. plantarum* CD033 and 3NSH using the method described in section 4.10.2.

In this experiment the linear DNA of the short integration cassette (5´3´cbh_CAT) was used.

Table 20: Reaction mixture for the transformation approach A and B.

Approach A [34]	
1.5 µl	ssDNA (~16 µg)
1 (1:10 dil.), 0.5, 2.6 µl	SSBnHis (0.2, 1, 5.2 µg)
x µl	SSB Storage buffer
10 µl	Final volume

Approach B [35, 36]	
1 (1:10 dil.), 1.5, 3.5 µl	ssDNA (1.1, 16, 37 µg)
2.6 µl	SSBnHis (5.2 µg)
x µl	SSB Storage buffer
10 µl	Final volume

Table 21: Reaction mixture for the transformation approach C and D.

Approach C	
1.5 µl	ssDNA (~16 µg)
1, 2, 4 µl	RecAnHis (2, 4, 8 µg)
1 µl	ATP (50 mM)
x µl	RecA assay buffer
10 µl	Final volume

Approach D	
1.5 µl	ssDNA (~16 µg)
2.6 µl	SSBnHis (5.2 µg)
4 µl	RecAnHis (8 µg)
1 µl	ATP (50 mM)
x µl	RecA assay buffer
10 µl	Final volume

4.10.1 Electrocompetent *L. plantarum* cells

25 ml MRS media were inoculated with *L. plantarum* CD033 or 3NSH and incubated overnight at 37°C under anaerobic condition. Main cultures (25 ml MRS+1% glycine) were inoculated to an optical density at 600 nm (OD_{600}) of 0.2 (measurement with Eppendorf BioPhotometer). The cells were grown at 37°C and anaerobic conditions until an OD_{600} of 0.6 was reached. The chilled cultures were harvested by centrifugation with 3220 x g and 4°C (Eppendorf Centrifuge 5810R). The cell pellets were washed twice with ice-cold sterile distilled water (1st step with 25 ml, 2nd with 12.5 ml). Afterwards the cells were washed in 2 ml ice-cold PEG_{1500} (30 % in water). Finally, the cells were resuspended in 250 µl 30 % PEG_{1500} and 40 µl aliquots were pipetted into fresh tubes. After freezing in liquid nitrogen, the cells were stored at - 80°C.

4.10.2 Transformation in *L. plantarum* by electroporation

A maximum of 10 to 15 µl of DNA (preferably high concentrated) was added to 40 µl electrocompetent *L. plantarum* CD033 and 3NSH cells. After 20 minutes of incubation on ice, the cells were transferred into ice-cold electroporation cuvettes and shocked using the electroporator Micropulser from BioRad (pulsed by 2 kV). After electroporation, the cells were revitalized in 1 ml MRS bouillon for 2 hours at 37°C without shaking. Then 100 µl of the cells and the remaining cells were plated on MRS agar-plates containing 10 µg/ml chloramphenicol (MRS-CM). The plates were incubated for 3 days at 37°C under anaerobic conditions.

4.11 Congo red-assay (Endoglucanase activity assay)

The Congo red forms a red complex with oligosaccarides containing a minimum of five monosaccarides linked by ß-1,3 or ß-1,4 bonds. When carboxy-methyl cellulose (CMC) is incorporated into agar, hydrolysis of CMC by endoglucanases results in products with less than five glucose units and therefore forms no complexes with Congo red. Endoglucanases produce a yellow halo zone against a red background which can be used to select endoglucanase producing strains.

The putative endoglucanase positive strains were spotted on LB-Amp- (*E. coli* strains) or MRS-CM-plates (*L. plantarum* strains) supplemented with 5 g/l CMC and incubated over night at 37°C. The grown colonies were washed from the agar plates. The plates were overlayed with Congo red solution (0.2% in water) and incubated for 30 minutes at room temperature on the rocker. Afterwards the plates were washed two times with 0.5 M NaCl solution for 30 minutes. After the washing steps the yellow halo zone should be visible.

4.12 Integration stability assay

The strain *L. plantarum* 3NSH A5-1 was inoculated in 8 ml of MRS media (without antibiotic) and incubated over night. Next, 100 µl of this over night culture were used for plating a 10^{-4} to 10^{-6} dilution onto MRS and MRS-CM plates while 10 µl of the over night culture were used for inoculation of fresh MRS media (again 8 ml). This inoculation and plating procedure was repeated for 20 days. The colony-forming unit (CFU) of the MRS plates and MRS-CM plates was determined after 48 hour incubation at 37°C under anaerobic conditions and compared to each other to see if the integration cassette is stable integrated.

5 Results

5.1 Construction of the pMS470nHis_ssb expression vector

The vector pMS470_ssb and pMS470nHis were double digested with *Nde*I and *Hind*III, to get the *ssb* fragment and the linear pMS470nHis vector, which were ligated to generate the plasmid pMS470nHis_ssb. The plasmid pMS470nHis_ssb was transformed into *E. coli* XL1-Blue cells by electroporation followed by plasmid isolation. The successful generation of the pMS470nHis_ssb was verified by double digestion with *Hind*III and *Nco*I. Agarose gel electrophoresis of the digested plasmid resulted in fragments of the expected sizes (3980 bp for the vector backbone and 665 bp for *ssbnHis* fragment, figure 33 lanes 2, 3, 5, 7, 8) or no DNA fragments at all (figure 33 lanes 4, 6, 9). A possible explanations for the empty lanes could be unsuccessful plasmid isolation.

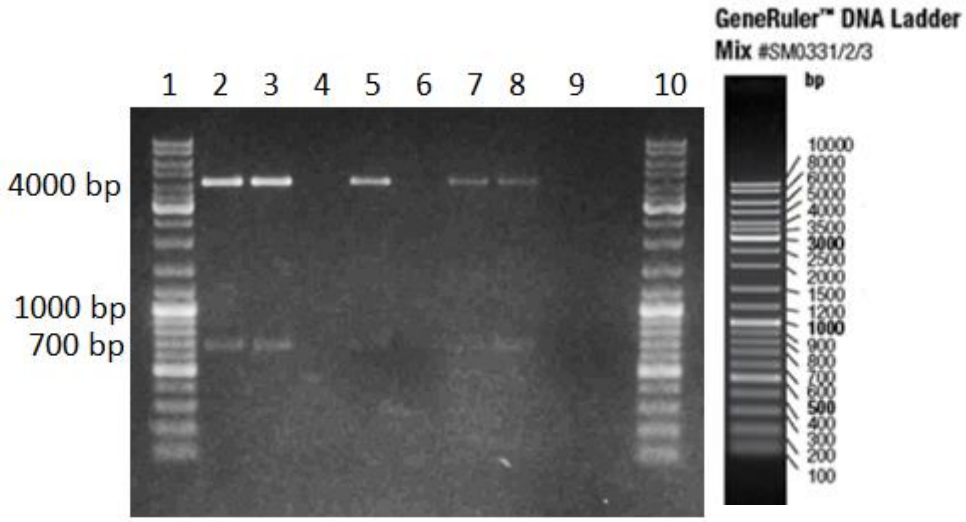

Figure 33: Cloning of the ssb fragment into pMS470nHis vector. *Double digestion of the generated pMS470nHis_ssb plasmid with HindIII and NcoI. Lane 1 and 10: GeneRuler™ DNA Ladder Mix; Lane 2-9: HindIII and NcoI digested plasmids pMS40nHis_ssb. The expected sizes are 3980 bp for the vector backbone and 665 bp for ssbnHis fragment.*

5.2 Construction of the pMS470nHis_recA expression vector

The plasmid pMS470_linker_recA was generated by double digestion (*Bgl*II and *HindIII*) and subsequent ligation of the plasmid pMS470_linker and the *recA* fragment (isolated from *L. plantarum* CD033 during the project laboratory, see project laboratory protocol "Recombination experiments with linear DNA in *Lactobacillus plantarum*" for details). The successful generation of the plasmid pMS470_linker_recA was verified by Colony PCR using the *recA* specific primer 137 and 166. Agarose gel electrophoresis of the Colony PCR is shown in figure 34. One transformant showed the expected size of the *recA* gene (1149 bp, figure 34 lane 25). Furthermore the sequence of the *recA* gene in the plasmid pMS470_linker_recA was investigated by sequence analysis (LGC genomics). The sequence obtained from LGC genomics matched the sequence of the initially isolated *recA* from *L. plantarum* CD033. The fact that the majority of the investigated colonies did not show the *recA* fragment could for example be caused by using too much cell material for the Colony PCR.

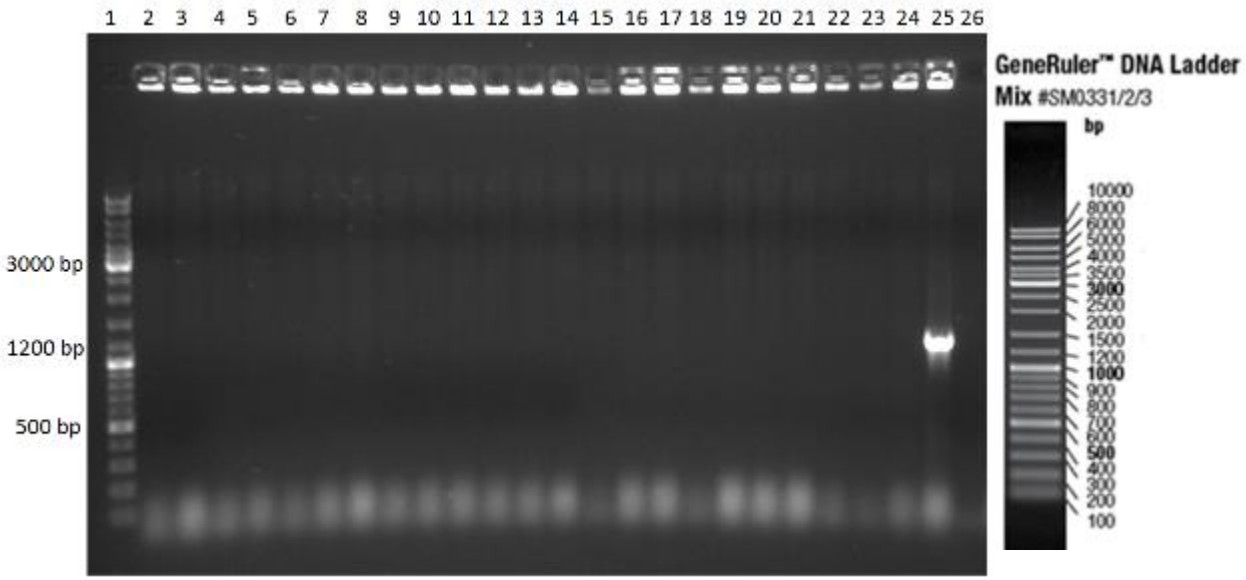

Figure 34: Cloning of the recA fragment into pMS470_linker vector. *Agarose gel after Colony PCR with the recA specific primers 166 and 137. Lane 1: GeneRuler™ DNA Ladder Mix; Lane 2-25: Transformants 1-24 presumably containing the plasmid pMS470_linker_recA. Lane 26: Mastermix. Expected size of the PCR product is 1149 bp.*

After a *Nde*I restriction site was introduced to the *recA* gene by Standard PCR (pMS470_linker_recA as template and primer 137 and 166 were used), the modified *recA* fragment was cloned into the vector pJET1.2 blunt to generate the plasmid pJET_recA. The successful generation of this plasmid was verified by double digestion with *Nde*I and *Hind*III. It is noteworthy that *Nde*I has an additionaly restriction site inside the *recA* gene (see figure 15). Therefore the double digestion resulted in fragments of approximately 4100 bp for the linearized plasmid, approximately 3000 bp for the vector backbone, approximately 900 bp for the first part of the recA gene and approximately 300 bp for the second part of the recA gene (figure 35). Furthermore the sequence of the *recA* gene in the plasmid pJET_recA was investigated by sequence analysis (LGC genomics). The sequence obtained from LGC genomics matched the sequence of the initially isolated *recA* from *L. plantarum* CD033.

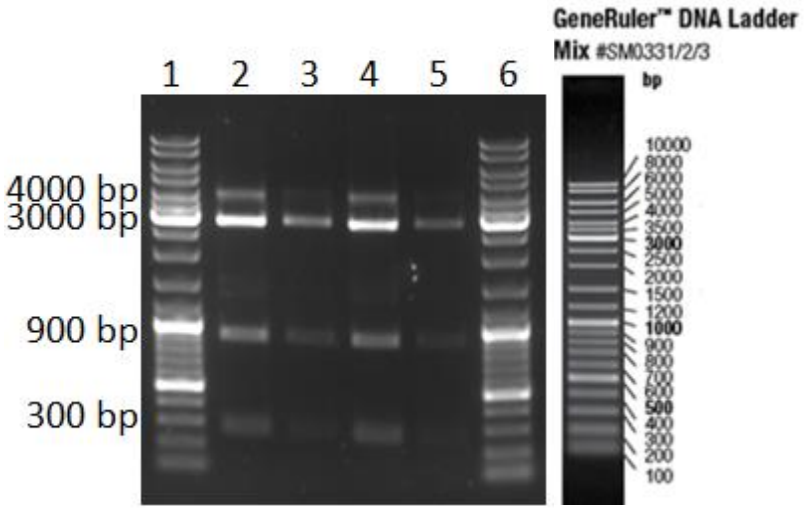

Figure 35: Cloning of the recA fragment into the pJET1.2 vector. *Double digestion of the generated plasmid pJET_recA with HindIII and NdeI. Lane 1 and 6: GeneRuler™ DNA Ladder Mix; Lane 2-5: digested pJET_recA. The expected sizes are 4123 bp for the linearized pJET_recA, 2980 bp for the vector backbone, 874 bp for the first part of the recA gene and 268 bp for the second part of the recA gene.*

Next the *recA* fragment was isolated from the pJET_recA plasmid by partial digestion with *Hind*III and *Nde*I (figure 36) and cloned into the vector pMS470nHis which was linearized with *Hind*III and *Nde*I to generate the plasmid pMS470nHis_recA.

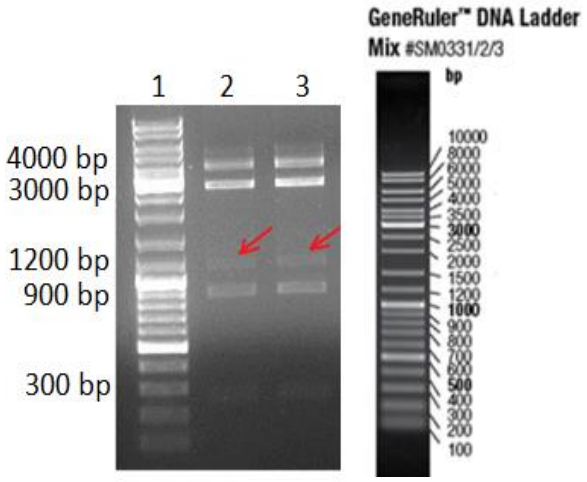

Figure 36: Partial digestion of the plasmid pJET_recA. *The plasmid pJET_recA was digested with HindIII and half of the recommended concentration of NdeI for 30 minutes at 37°C. Lane 1: GeneRuler™ DNA Ladder Mix; Lane 2-3: digested pJET_recA. The expected sizes are 4123 bp for the linearized pJET_recA, 2980 bp for the vector backbone, 1143 bp for the full length recA gene, 874 bp for the first part of the recA gene and 268 bp for the second part of the recA gene. The isolated bands are marked by red arrows.*

The successful generation of the plasmid pMS470nHis_recA was verified by double digestion with *Nde*I and *Hind*III which resulted in fragments of approximately 4000 bp for the vector backbone, approximately 900 bp for the first part of the recA gene and approximately 300 bp for the second part of the recA gene (figure 37). Furthermore the sequence of the *recA* gene in the plasmid pMS470nHis_recA was investigated by sequence analysis (LGC genomics). The sequence obtained from LGC genomics matched the sequence of the initially isolated *recA* from *L. plantarum* CD033.

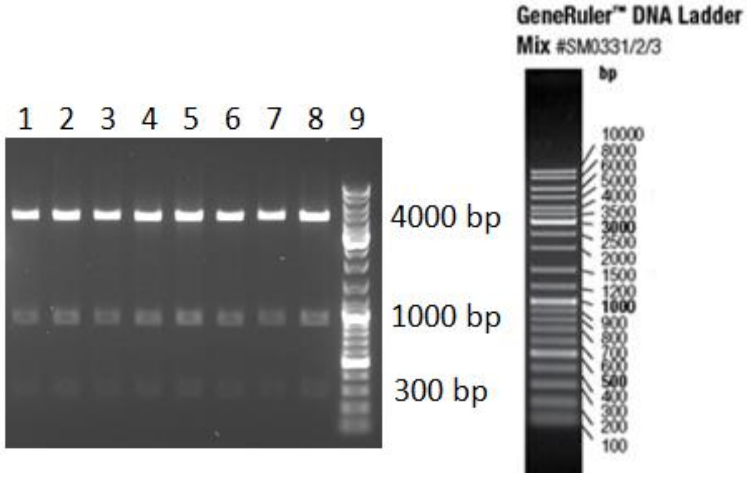

Figure 37: Cloning of the recA fragment into the pMS470nHis_recA. *Double digestion of the generated plasmid pMS470nHis_recA with HindIII and NdeI. Lanes 1-8: digested pMS470nHis_recA; Lane 9: GeneRuler™ DNA Ladder Mix. The expected sizes are 4039 bp for the vector backbone, 874 bp for the first part of the recA gene and 268 bp for the second part of the recA gene.*

5.3 Heterologous expression and purification of the His_6-tagged SSB and RecA

In order to investigate the expression of the His_6-tagged SSB and RecA in the strains *E. coli* BL21DE3 [pMS470nHis_ssb], *E. coli* BL21DE3 [pET28b_ssb] and *E. coli* BL21DE3 [pMS470nHis_recA], the experiment was first carried out in 5 ml main cultures after IPTG induction (0.1 mM) at 28 °C overnight (as described in part 4.4). After sonication the soluble fraction and the insoluble fraction (followed by resuspension in 1 ml Lysis Buffer) were analysed on a SDS polyacrylamide gel. The sizes of the His_6-tagged SSB and the RecA proteins differ significantly from the theoretical sizes (SSBnHis= 23.8 kDa and RecAnHis= 42.9 kDa, as calculated by the ExPASy ProtParam tool using the SSBnHis and RecAnHis protein sequences indicated in the appendix). A possible explanation of this unexpected molecular weight shift could be posttranslational modifications by the used *E. coli* BL21DE3 strain [37]. Another possible explanation for the molecular weight shift could be inappropriate denaturation of the proteins which would affect their electrophoretic mobility. Furthermore a mistake during the cloning procedure for RecAnHis (a primer was used that removed the stop codon) resulted in the deletion of the stop codon. Fortunately another stop codon was present shortly after the original stop codon. Therefore the resulting RecA protein has additional amino acids at the C-terminal end (KLGCFGG) which increases its theoretical size to 43.5 kDa. However, this change of the theoretical molecular weight is not sufficient to explain the observed shift. The SDS polyacrylamide gel compared to the Westernblot are shown in figure 38 (SSBnHis). For the RecAnHis the SDS polyacrylamide gel and the Westernblot are shown in figure 39 and 40.

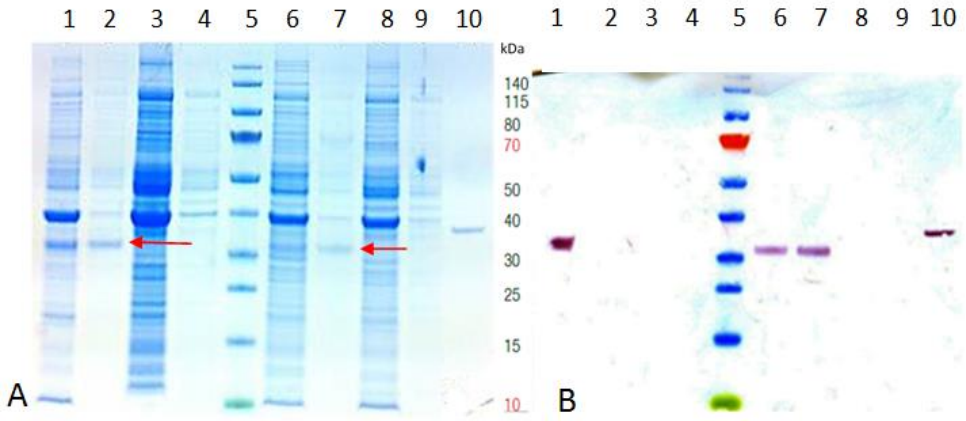

Figure 38: Heterologous expression of SSBnHis. *The indicated fractions were subject to SDS PAGE (4-12 % Bis-Tris, MES buffer) (A) or Westernblot with specific antibodies targeting the His_6- tag (B). Lane 1: E. coli BL21DE3 [pMS470nHis_ssb] insoluble fraction; Lane 2: E. coli BL21DE3 [pMS470nHis_ssb] soluble fraction, Lane 3: E. coli BL21DE3 [pMS470Δ8] insoluble fraction; Lane 4: E. coli BL21DE3 [pMS470Δ8] soluble fraction; Lane 5: PageRuler™ Prestained protein ladder; Lane 6: E. coli BL21DE3 [pET28b_ssb] insoluble fraction; Lane 7: E. coli BL21DE3 [pET28b_ssb] soluble fraction; Lane 8: E. coli BL21DE3 [pET28b] insoluble fraction; Lane 9: E. coli BL21DE3 [pET28b] soluble fraction; Lane 10: positive control purified CelAc_nHis (N-terminal His_6-tagged endoglucanase). The corresponding bands in the SDS PAGE are indicated by a red arrow.*

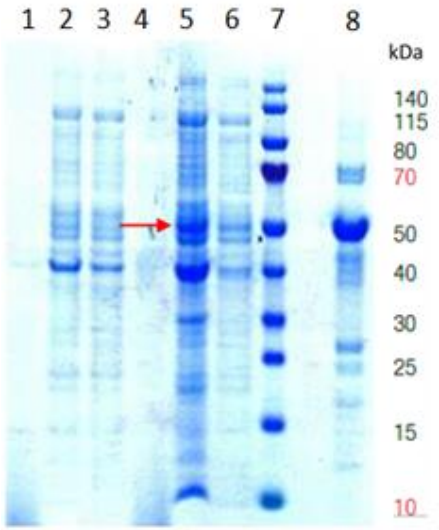

Figure 38: Heterologous expression of RecAnHis*. The indicated fractions were subject to SDS PAGE (4-12 % Bis-Tris, MES buffer). Lane 1: E. coli BL21DE3 [pMS470Δ8] culture supernatant; Lane 2: E. coli BL21DE3 [pMS470Δ8] insoluble fraction; Lane 3: E. coli BL21DE3 [pMS470Δ8] soluble fraction; Lane 4: E. coli BL21DE3 [pMS470nHis_recA] culture supernatant; Lane 5: E. coli BL21DE3 [pMS470_recAnHis] insoluble fraction; Lane 6: E. coli BL21DE3 [pMS470nHis_recA] soluble fraction, Lane 7: PageRuler™ Prestained protein ladder, 8: positive control purified CelA_cHis (C-terminal His_6-tagged endoglucanase).*

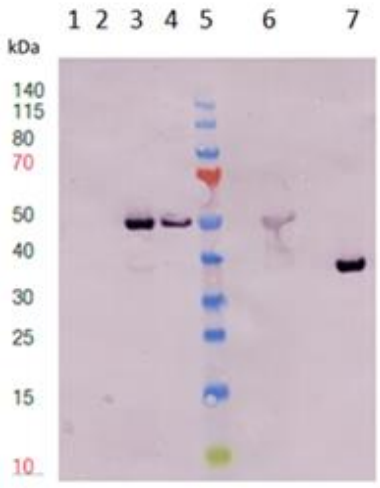

***Figure 40: Heterologous expression of RecAnHis**. The indicated fractions were subject to Westernblot with specific antibodies targeting the His_6-tag. Lane 1: E. coli BL21DE3 [pMS470Δ8] insoluble fraction; Lane 2: E. coli BL21DE3 [pMS470Δ8] soluble fraction; Lane 3: E. coli BL21DE3 [pMS470nHis_recA] insoluble fraction; Lane 4: E. coli BL21DE3 [pMS470nHis_recA] soluble fraction, Lane 5: PageRuler™ Prestained protein ladder; Lane 6: positive control purified CelA_cHis (C-terminal His_6-tagged endoglucanase); Lane 7: positive control purified CelAc_nHis (N-terminal His_6- tagged endoglucanase). The corresponding bands in the SDS PAGE are indicated by a red arrow.*

All further experiments have been performed using the strain *E. coli* BL21DE3 [pMS470nHis_ssb] and *E. coli* BL21DE3 [pMS470nHis_recA]. As indicated in figure 38 both strains (*E. coli* BL21DE3 [pMS470nHis_ssb], *E. coli* BL21DE3 [pET28b_ssb]) heterologously express the N-terminally tagged SSB. Even though there is no SSB visible in the Westernblot of the soluble fraction of the *E. coli* BL21DE3 [pMS470nHis_ssb] position (Figure 38 A red arrows). A possible explanation for the missing Western blot band could be an air bubble at the height of the target protein. Taken together all investigated strains heterologously express SSBnHis or RecAnHis respectively.

In order to acquire sufficient amounts of the recombinant proteins the expression was performed in 100 ml main cultures. The heterologously expressed proteins were then purified using a Ni-NTA sepharose columns (GE Healthcare 17-5318). The purification was optimized by testing several imidazole concentrations in the Wash Buffer. The best results were obtained by using 2 times 1 CV (column volume) 30 mM imidazole Wash Buffer followed by 2 times 1 CV 40 mM imidazole wash buffer (purification protocol as described in part 4.5).

All fractions were loaded on a SDS polyacrylamide gel to verify the successful purification (figure 41 and 42). It is noteworthy that the SDS PAGE gels of figure 38 and 39 use a different buffer system compared to figure 41 and 42. Due to the different pH of these buffer systems (pH 7.3 for the MES buffer and pH 8.6 for the Tris-glycine buffer) the mobility of the proteins is changed, which results in the different sizes of the proteins shown in the respective figures.

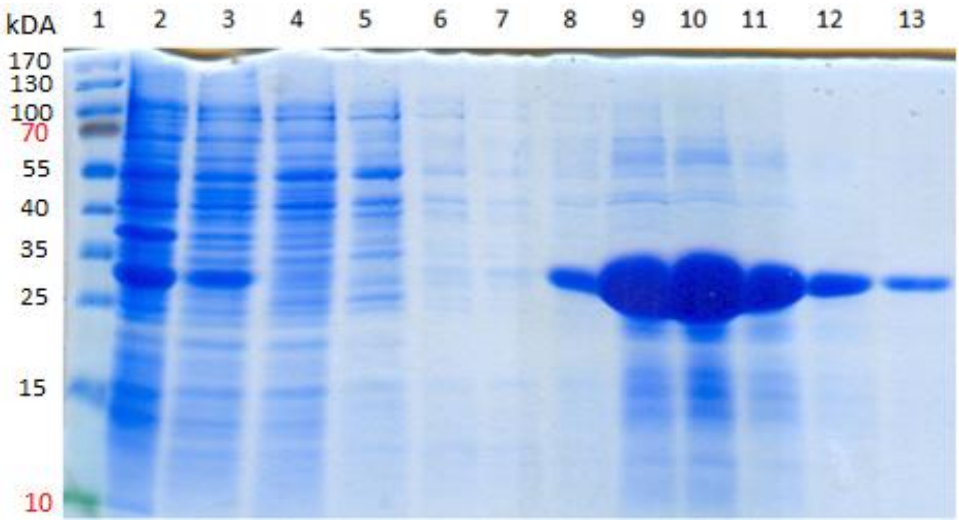

Figure 41: Purification of SSBnHis. *12 % Tris-glycine SDS-PAGE (Tris-glycine buffer) after Ni-NTA sepharose column purification of the protein SSBnHis. Lane 1: PageRuler™ Prestained protein ladder; Lane 2: E. coli BL21DE3 [pMS470nHis_ssb] insoluble fraction; Lane 3: flow-through; Lanes 4-7: wash fractions; Lanes 8-13: elution fractions.*

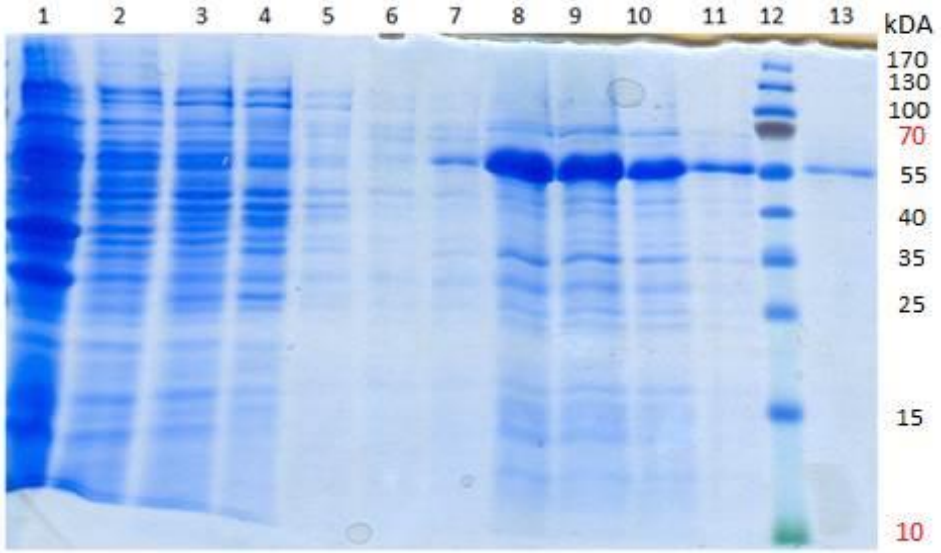

Figure 42: Purification of RecAnHis. *12 % Tris-glycine SDS-PAGE (Tris-glycine buffer) after purification of the protein RecAnHis. Lane 12: PageRuler™ Prestained protein ladder; Lane 1: E. coli BL21DE3 [pMS470nHis_recA] insoluble fraction; Lane 2: flow-through fraction; Lanes 3-6: wash fractions; Lanes 7-11, 13: elution fractions.*

Despite the fact that the acquired elution fractions (figure 40 lanes 8-13 and figure 41 lanes 7-13) were not completely pure, they showed a good amount of the desired protein. The elution fractions were pooled, rebuffered to the corresponding storage buffer and concentrated by centrifugation using the Vivaspin® 4 column (PES membrane, 10000 MWCO, Satorius stedim biotech). The protein concentration was measured spectrophotometrically at a wavelength of 280 nm using NanoDrop 2000c (Thermo Scientific, extinction coefficient for SSBnHis is 14440 $M^{-1}*cm^{-1}$ and for RecAnHis 20400 $M^{-1}*cm^{-1}$ as calculated by the ExPASy ProtParam tool using the SSBnHis and RecAnHis protein sequences indicated in the appendix). The resulting concentration of the SSBnHis protein solution was 1.99 mg/ml and for the RecAnHis protein solution 1.93 mg/ml.
Taken together, the heterologous expression of the recombinant proteins of SSBnHis and RecAnHis as well as the purification using a Ni-NTA sepharose column were successful.

5.4 Generation of linear ssDNA from the short integration cassette 5´3´cbh_CAT

In order to obtain linear ssDNA either the forward or the reverse primer was used in 100-fold excess in a PCR with the plasmid pMS470_5´3´cbh_CAT as template using different temperature profiles. The resulting PCR products were investigated by agarose gel electrophoresis (figure 43-44).
Excess of either the forward primer (figure 43, lane 2) or the reverse primer (figure 43, lane 4) of the PCR using the temperature profile A resulted in bands of approximately 2400 bp. This size matches the theoretical size of the expected fragment. However the electrophoretic mobility of large ssDNA is reported to be different to the mobility of dsDNA [38] which has to be considered while interpreting the results. Additionally bands at about 5000 bp were also visible. These bands could be caused by the inappropriate temperature profile used in this experiment. Accidentally a temperature profile suitable for the DreamTaq polymerase was used for the PCR with the Phusion polymerase. The PCR mastermixes (all components except template DNA) were also investigated but showed no noteworthy bands (figure 43; lanes 3 and 5).

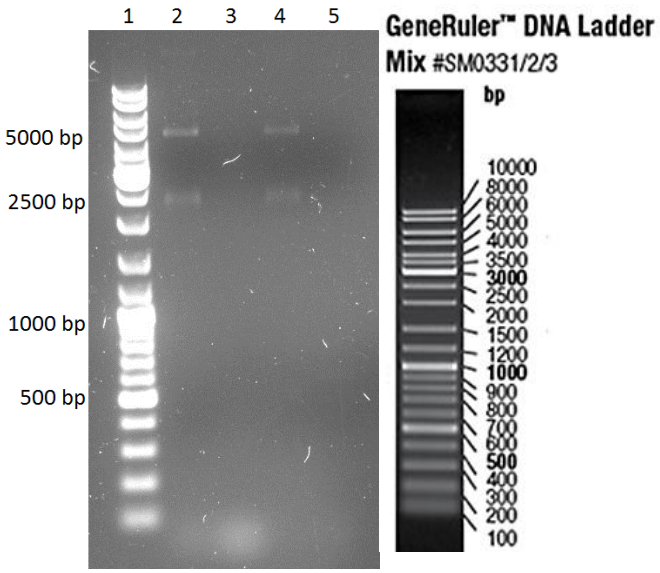

Figure 43: ssDNA generated by excess addition of one primer. *Agarose gel of ssDNA generated by PCR using the plasmid pMS470_5´3´cbh_CAT as template and excess of either the forward or reverse primers using the temperature profile A. Lane 1: GeneRuler™ DNA Ladder Mix; lane 2: ssDNAfwd (5´3´cbh_CAT); lane 3: PCR mastermix for ssDNAfwd; lane 4: ssDNArev (5´3´cbh_CAT); lane 5: PCR mastermix for ssDNArev. For all reactions the theoretical size of the amplified fragment is approximately 2400 bp.*

Excess of either the forward primer (figure 44, lanes 2-5) or the reverse primer (figure 44, lanes 7-10) of the PCR using the temperature profile B resulted in bands of approximately 2400 bp which matches the theoretical size of the expected fragment. Additional unspecific bands below 1000 bp were also visible in the reaction with the forward primers in 100 fold excess (figure 44 lanes 2-5). These unspecific bands were even present in the mastermix without template DNA which indicates primer dimer formation (figure 44 lane 6). The discrepancies of the band intensities in lane 8 and 9 of figure 44 suggest that the template was accidentally applied twice to the reaction mixture shown in lane 9. No noteworthy bands are visible in the mastermix (all components except template DNA) of the reactions using the reverse primer in 100 fold excess (figure 44 lane 11).

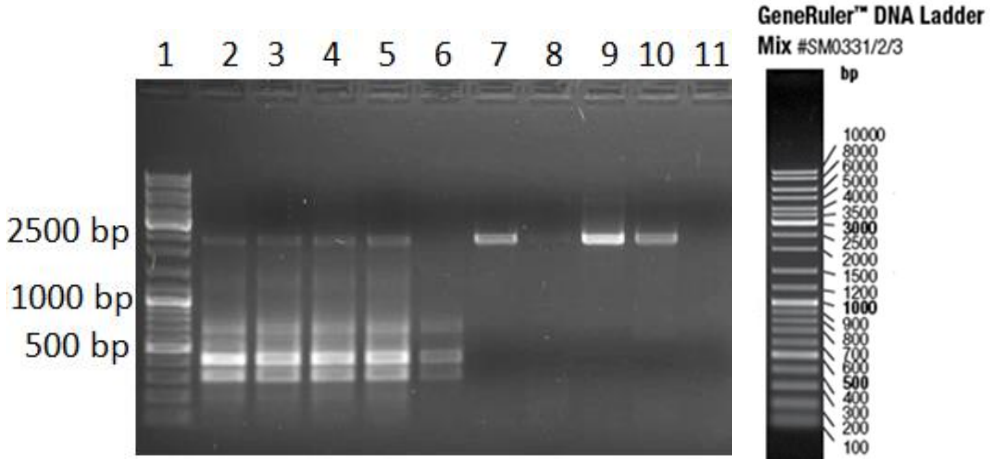

Figure 44: ssDNA generated by excess addition of one primer. *Agarose gel of ssDNA generated by PCR using the plasmid pMS470_5´3´cbh_CAT as template and excess of either the forward or reverse primer using the temperature profile B. Lane 1: GeneRuler™ DNA Ladder Mix; lane 2-5: ssDNAfwd (5´3´cbh_CAT); lane 6: PCR mastermix for ssDNAfwd; lane 7-10: ssDNArev (5´3´cbh_CAT); lane 11: PCR mastermix for ssDNArev. For all reactions the theoretical size of the amplified fragment is approximately 2400 bp.*

To generate sufficient quantity of the putative ssDNA for the subsequent transformation experiments, multiple 50 µl PCR reactions using the temperature profile A were pooled and concentrated via ethanol precipitation (see part 4.6.1). The DNA concentration was measured by NanoDrop 2000c (Thermo Scientific) at 260 nm. The resulting concentration of the putative ssDNAfwd was 10.7 µg/µl and for the putative ssDNArev 10.5 µg/µl.

5.5 Gel shift assay

The gel shift assay was performed to verify the binding ability of the heterologously expressed SSB and RecA to DNA. In the first approach the DNA binding capacity of SSBnHis and RecAnHis were compared to the non DNA binding protein ADHnHis (alcohol dehydrogenase isolated from *Ralstonia eutropha* H16 and kindly provided by Zalina Magomedova) by incubating increasing concentrations of the proteins with putative ssDNA generated by 100-fold excess of either the forward (figure 45 lanes 2-8) or the reverse primer (figure 45 lanes 9-15). The DNA generated using the forward primer in 100-fold excess showed the same unspecific bands as described earlier. The addition of SSBnHis (figure 45 lanes 3-4 and lanes 10-11) clearly resulted in a shift of the DNA compared to unloaded DNA (figure 45 lane 2 and lane 9). The shift of the DNA band at around 2400 bp did not increase with higher SSBnHis concentration. However, the addition of SSB resulted

in the formation of a “cloud” at around 4000 bp and the shift of this cloud further increased with increasing SSB concentration. The addition of RecAnHis resulted in a protein concentration dependant decrease of the band intensities at approximately 2400 bp (figure 45 lanes 5-6 and lanes 12-13). This decrease could be the result of the filament formation of the RecAnHis monomers around the DNA which could lead to huge molecules that cannot properly penetrate the agarose gel. Such huge protein/DNA complexes could be stuck in the slot of the agarose gel as seen in figure 45 lanes 5-6 and lanes 12-13. Another possibility would be a nuclease contamination of the RecAnHis protein solution which leads to the degradation of the DNA. Incubation of DNA with ADHnHis resulted in no significant changes of the DNA bands at approximately 2400 bp (figure 45 lanes 7-8 and lanes 14-15). However, “clouds” similar to the ones observed with SSBnHis, but with much lower intensity are visible and indicate a minor protein/DNA complex formation. Because ADH has no DNA binding properties, it is possible that the ADHnHis protein solution still contained minimal residues of DNA binding proteins that remained after the ADHnHis purification.

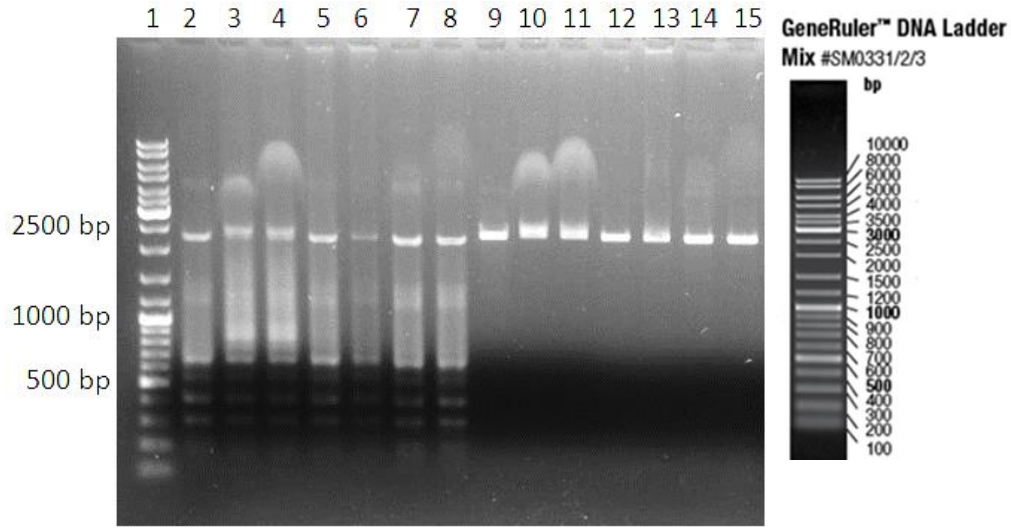

Figure 45: Gel shift assay using SSBnHis, RecAnHis and ADHnHis with ssDNA. *Agarose gel of putative ssDNA generated by 100-fold excess of either the forward (lanes 2-8) or the reverse primers (lanes 9-15) incubated with different concentrations of SSBnHis, RecAnHis and ADHnHis (alcohol dehydrogenase). Lane 1: GeneRuler™ DNA Ladder Mix; lanes 2 and 9: unloaded DNA; lanes 3 and 10: DNA with 2 µg SSBnHis; lanes 4 and 11: DNA with 6 µg SSBnHis; lanes 5 and 12: DNA with 2 µg RecAnHis; lanes 6 and 13: DNA with 6 µg RecAnHis; lanes 7 and 14: DNA with 2 µg ADHnHis; lanes 8 and 15: DNA with 6 µg ADHnHis. For all reactions the theoretical size of the amplified fragment is approximately 2400 bp.*

To further assess the DNA binding capacity of SSBnHis and RecAnHis compared to ADHnHis, the proteins were incubated with dsDNA generated by Standard PCR (primer 138 and 139, see 4.1.3 for details). Incubation of the dsDNA with SSBnHis resulted in a protein concentration dependant shift of the DNA bands (figure 46 lanes 2-3) compared to unloaded DNA (figure 46 lane 1). However this shift is less pronounced than the shift of the putative ssDNA incubated with SSBnHis shown in figure 46, suggesting a preference of SSBnHis for ssDNA. The addition of RecAnHis to the dsDNA resulted in a protein concentration dependant decrease of the band intensities (figure 46 lanes 4 and 5). As mentioned before this could be caused by the reduced exposure of free DNA to the ethidium bromide or nuclease contamination of the RecAnHis protein solution. Unfortunately the addition of ADHnHis to dsDNA cannot be assessed because some of the sample of lane 6 accidentally spilled over to lane 7 during the loading of the gel.

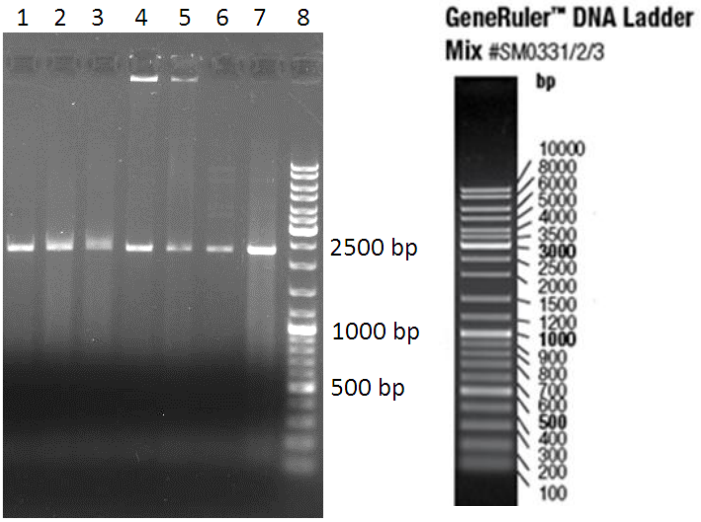

Figure 46: Gel shift assay using SSBnHis, RecAnHis and ADHnHis with dsDNA. *Agarose gel ofdsDNA generated by Standard PCR incubated with different concentrations of SSBnHis, RecAnHis and ADHnHis (alcohol dehydrogenase). Lane 1: unloaded DNA; lane 2: DNA with 2 µg SSBnHis; lane 3: DNA with 6 µg SSBnHis; lane 4: DNA with 2 µg RecAnHis; lane 5: DNA with 6 µg RecAnHis; lane 6: DNA with 2 µg ADHnHis; lane 7: DNA with 6 µg ADHnHis; lane 8: GeneRuler™ DNA Ladder Mix. For all reactions the theoretical size of the amplified fragment is approximately 2400 bp.*

5.6 DNA/protein loading and transformation into *L. plantarum*

In order to integrate the short linear integration cassette (5´3´cbh_CAT) into the genome of *L. plantarum* linearized dsDNA (5 µg dsDNA without protein) or ssDNA in the presence of the DNA binding proteins SSBnHis and RecAnHis (summarized in table 20 and 21) was used. Additionally, integration experiments with ssDNA (0.5 µg, 5 µg, 16 µg and 37 µg) in the absence of DNA binding proteins were performed. The experiments were carried out with the ssDNA generated by asymmetric PCR using the forward (primer 138) or the reverse primer (primer 139) in 100-fold excess with the plasmid pMS470_5´3´cbh_CAT (cccDNA) as template. The transformation was performed into the *L. plantarum* strains CD033 and 3NSH.

The integration experiments using ssDNA did not result in any chloramphenicol resistant colonies. Only the experiment transforming dsDNA into the *L. plantarum* strain 3NSH resulted in one single colony. This colony was again streaked onto a MRS-CM plate to confirm the chloramphenicol resistance. Further the cells were assessed by microscopic examination to confirm the rod-shaped morphology of the bacterium.

To verify the integration of the 5´3´cbh_CAT cassette into the genome of *L. plantarum,* PCRs with different primer combinations were performed and investigated using agarose gel electrophoresis. The position of the different primer combinations are shown in figure 47 and the primer names and sequences are listed in table 5. The fragment sizes of the different primer combinations are summarized in table 22.

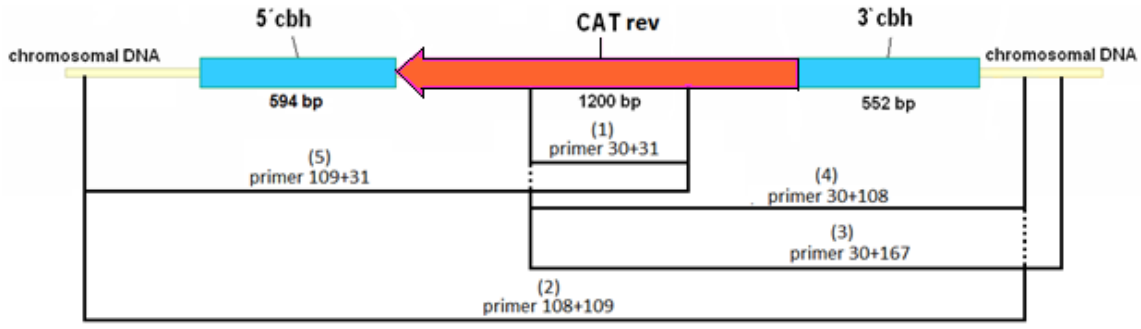

Figure 47: Schematic representation of the short integration cassette 5´3´cbh_CAT after integration into the host genome. *The primer combinations used to confirm the integration are indicated by lines and the abbreviations are shown in brackets.*

Table 22: Fragment sizes of primer combination 1-5

Primer combination	Fragment size (bp)
(1) 30+31	~500
(2) 108+109	~2700
(3) 30+167	~1500
(4) 30+108	~1400
(5) 109+31	~1750

The primer combination 1 (figure 48, lane 2) resulted in a band at the expected size of 500 bp which resembles a fragment within the *CAT* sequence. The primer combination 2 (figure 48, lane 4) showed multiple bands. The most prominent band has a size of approximately 1500 bp. This band could be an indication that the used *L. plantarum* strain possesses multiple copies of the wild type *cbh* gene and its adjacent gene regions, which has a size of approximately 1500 bp and would also be amplified by the used primers. Another possible explanation for the high intensity of this band could be the presence of wild type *L. plantarum* during the genomic DNA isolation. The faint band at 2700 bp matches the expected band size of the primer combination 2. The third band which is located at a size of approximately 4000 bp could be an indication for consecutive recombination which could lead to a product of higher molecular weight. Another possible explanation for the largest band could be unspecific primer interactions that result in the observed product. The primer combination 3 (figure 48, lane 6) resulted in a band of approximately 1500 bp which resembles the expected size. Additionally this primer combination generated a large fragment at about 7000 bp which again could be an indication for multiple consecutive recombination events or unspecific primer interactions. The primer combination 4, which amplifies a similar fragment as the primer combination 3, showed a band at the expected size of 1400 bp (figure 48, lane 8). Also unexpected bands of the sizes 2500 bp and 7000 bp are visible. Like before these bands could be the result of multiple recombination events or unspecific primer binding. The primer combination 5 (figure 48 lane10) resulted in a band of approximately 1750 bp which matches the expected band size. The related PCR mastermixes (all components except template DNA) were also investigated but showed no noteworthy bands (figure 48 lanes 3, 5, 7, 9 and 11).

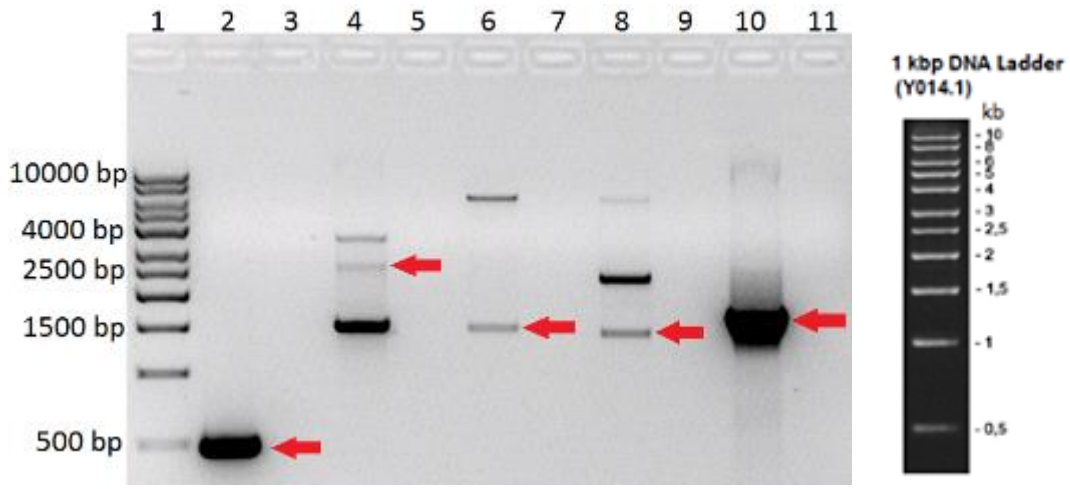

Figure 48: Verification of the integration of 5´3´cbh_CAT. *Agarose gel (1%) of PCR using different primer combinations to verify the integration of the short integration cassette (5´3´cbh_CAT). Lane 1: 1 kbp DNA ladder (Roth); lane 2: primer combination 1 (30+31, expected size 500 bp); lane 4: primer combination 2 (108+109, expected size 2700 bp); lane 6: primer combination 3 (30+167, expected size 1500 bp); lane 8: primer combination 4 (30+108, expected size 1400 bp); lane 10: primer combination 5 (109+31, expected size 1750 bp); lanes 3, 5, 7, 9 and 11: respective PCR mastermixes. The expected fragments are indicated by red arrows.*

5.7 Construction of the Flp/*frt* based integration cassette

The Flp/*frt* based integration cassette was generated in several steps (see figure 49).

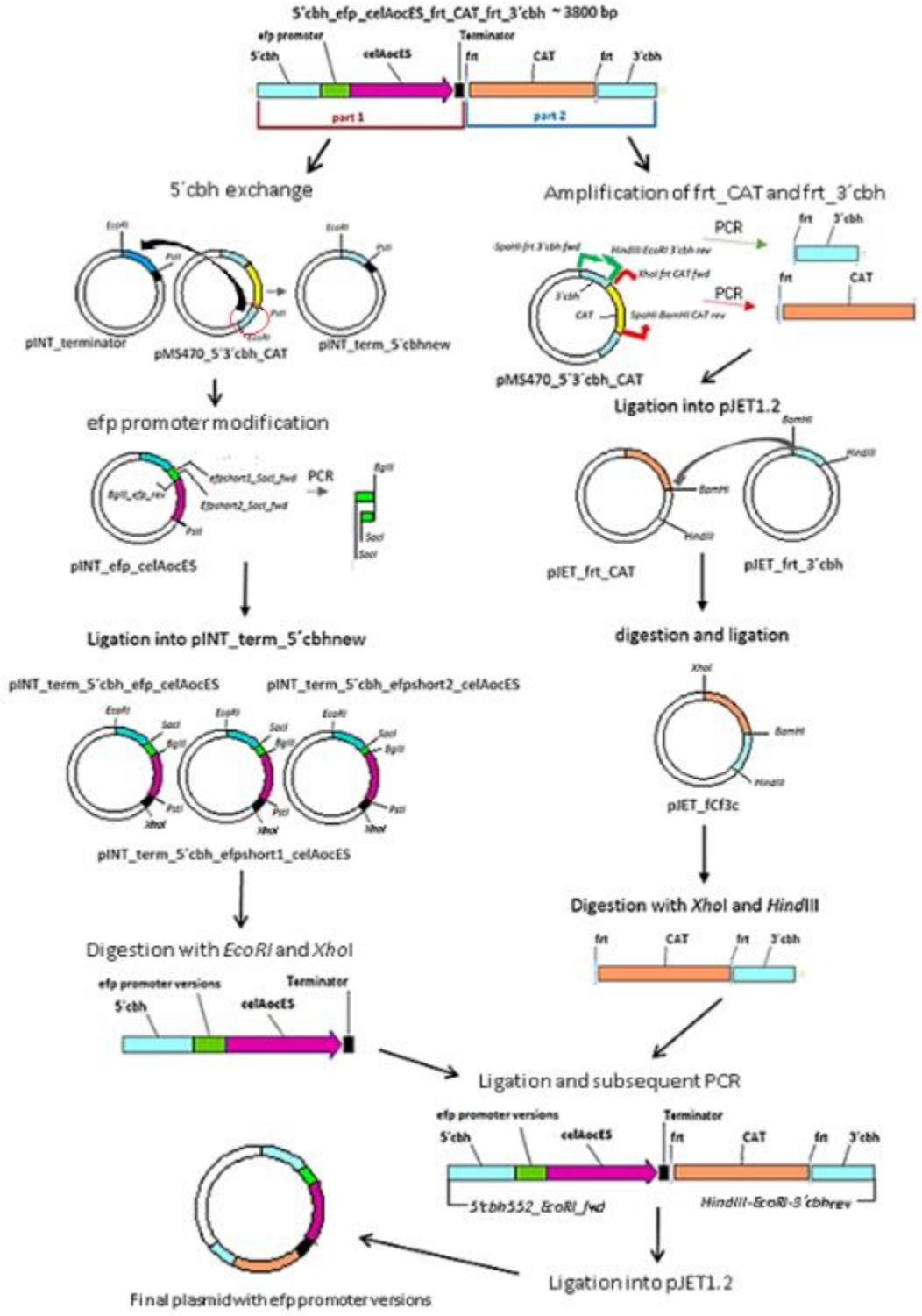

Figure 49: Schematic representation of the cloning strategy used to generate the Flp/frt based integration cassette.

Cloning of the 5´cbh_efp_celAocES construct (part 1)

The *efp* promoter used for the construction of the Flp/*frt* based integration cassette contained remains of the gene encoding the ribosomal protein L27 and remains of the *CAT* sequence. These sequences have been removed by Standard PCR using the primers 189 and 188 (for efpshort1) as well as 190 and 188 (for efpshort2, see table 4 primer list for details). The resulting PCR products were cloned into the plasmid pINT_efp_celAocES, double digested with *Sac*I and *Bgl*II, ligated and transformed into electrocompetent *E. coli* XL1-Blue. After transformation, 8 colonies of each promoter modification were picked for plasmid isolation and control digestion with *Sac*I and *Bgl*II (figure 50). The plasmids isolated from colony 5 (pINT_efpshort1_celAocES, figure 48, lane 5) and colony 15 (pINT_efpshort2_celAocES, figure 50, lane 15) showed the expected fragment sizes of the shortened promoters (210 bp for efpshort1 and 150 bp for efpshort2 compared to 265 bp for the original *efp*, figure 50 red arrows). Additionally the vector backbone is visible at about 7100 bp. The inconsistent fragment sizes of the different transformants of both promoter modifications suggests a cross contamination during the preparation of the plasmids. The sequence of the plasmids isolated from these colonies (pINT_efpshort1_celAocES from colony 5 and pINT_efpshort2_celAocES from colony 15) was verified using sequence analysis (LGC genomics GmbH) and used for further cloning steps. The sequences obtained from LGC genomics matched the sequence of the *efp* promoter in the plasmid pINT_efp_celAocES (CD-Lab Graz strain collection #113).

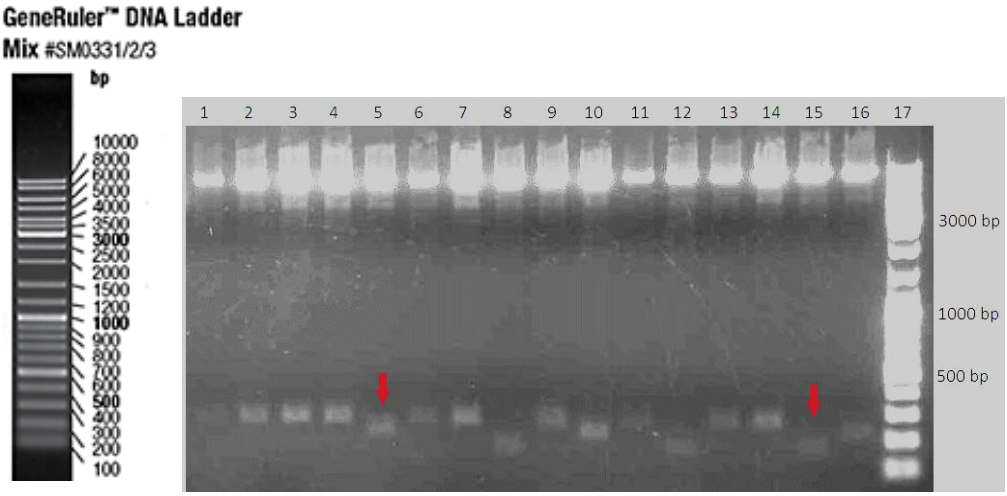

Figure 50: Modification of the efp promoter regions. *Agarose gel (1%) of the digested modified efp promoter plasmids. Lanes 1-8: plasmid 1-8 of pINT_efpshort1_celAocES digested with SacI and BglII (expected fragment size 7120 and 210 bp); lanes 9-16: plasmid 1-8 of pINT_efpshort2_celAocES digested with SacI and BglII (expected fragment size 7190 bp and 150 bp); 17: GeneRuler™ DNA Ladder Mix. The fragments corresponding to the expected sizes of the efp promoter versions from the further used plasmids are indicated by red arrows.*

The plasmid pINT_term_5´cbhnew was generated by double digestion of the plasmids pMS470_5´3´cbh_CAT and pINT_terminator (see figure 22 in part 4.7) using the restriction enzymes *Eco*RI and *Pst*I followed by ligation and transformation into *E. coli* XL1-Blue cells. The transformants were screened for the *5´cbh* gene by Colony PCR using the Primers 122 and 123. Four transformants showed a band with the expected size of the *5`cbh* gene (613 bp, figure 51 lane 2, 4, 8 and 16). The plasmid pMS470_5´3´cbh_CAT was used as a positive control and also showed a band of the expected size (figure 51 lane 17). The screened colonies that showed no bands indicate that the plasmid isolation was unsuccessful (figure 51 lanes 3, 5-7 and 9-15). The PCR mastermix (all components except template DNA) was also investigated but showed no noteworthy bands (figure 51 lane 18).

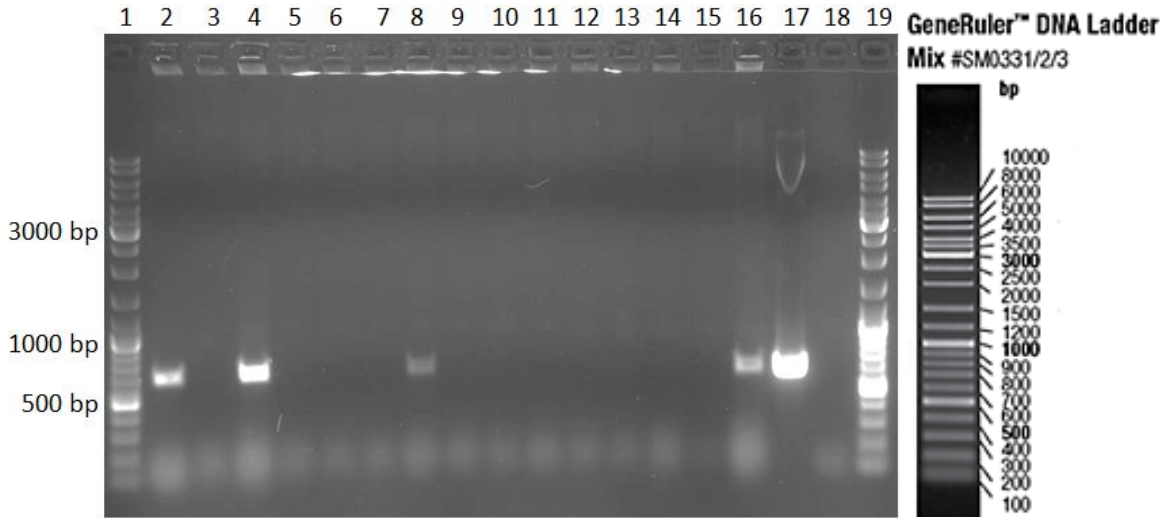

Figure 51: Cloning of the pINT_term_5´cbhnew plasmid. *Agarose gel (1%) after Colony PCR using the 5´cbh specific primers 122 and 123. Lane 1+19: GeneRuler™ DNA Ladder Mix; lane 2-16: transformant with the putative plasmid pINT_term_5´cbhnew; lane 17: positive control (pMS470_5´3´cbh_CAT); lane 18: mastermix. The 5´cbh fragment size is 613 bp.*

Next the efp_celAocES fragment or the respective versions with the shortened promoters (from the plasmids pINT_efp_celAocES, pINT_efpshort1_celAocES and pINT_efpshort2_celAocES) were cloned into the plasmid pINT_term_5´cbhnew, by double digestion with *Pst*I and *Sac*I. The resulting plasmids were named pINT_term_5´cbh_efp_celAocES, pINT_term_5´cbh_efpshort1_celAocES and pINT_term_5´cbh_efpshort2_celAocES and transformed into electrocompetent *E. coli* XL1-Blue. The transformants were spotted onto LB-Amp-CMC plates to verify endoglucanase activity of the introduced gene *celAocES* by the Congo red-assay (figure 52). The endoglucanase positive transformants number 3 of the pINT_term_5´cbh_efp_celAocES transformation, number 5 of the pINT_term_5´cbh_efpshort1_celAocES transformation and number 1 of the pINT_term_5´cbh_efpshort2_celAocES transformation were used for plasmid isolation.

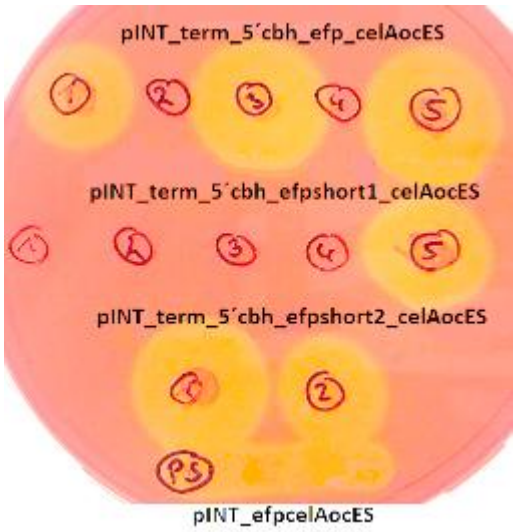

Figure 52: Congo red-assay of the 5´cbh_efpcelAocES transformants. *Resistant colonies of transformation experiments using the plasmids pINT_term_5´cbh_efp_celAocES, pINT_term_5´cbh_efpshort1_celAocES and pINT_term_5´cbh_efpshort2_celAocES were spotted onto LB-Amp-CMC plates. The yellow halos indicate the endoglucanase activity. E. coli XL1-Blue [pINT_efpcelAocES] is used as endoglucanase positive control (PS). The numbers mark the different transformants of the indicated plasmid.*

Cloning of the frt_CAT_frt_3´cbh construct (part 2)

In order to generate the frt_CAT_frt_3´cbh moiety of the Flp/*frt* based integration cassette the *frt* sites had to be attached to the *CAT* and *3´cbh* sequences and finally joint together. To this purpose the *CAT* and *3´cbh* sequences of the plasmid pMS470_5´3´cbh_CAT were used as template for a Standard PCR using the primers 180 + 181 for the frt_CAT fragment and 182 + 183 for the frt_3´cbh fragment. This PCR reaction resulted in the frt_CAT fragment (approximately 1200 bp) and the frt_3´cbh fragment (approximately 600 bp) shown in figure 53 A. After agarose gel purification these fragments were cloned into the pJET1.2 blunt to generate the plasmids pJET_frt_CAT and pJET_frt_3´cbh (figure 54 A and B). The correct ligation of these plasmids was confirmed by double digestion with *Xho*I and *Bam*HI for the frt_CAT construct (figure 53 B lanes 1-6) and with *Bam*HI and *Hind*III for the frt_3´cbh construct (figure 53 B lanes 7-12) which resulted in the expected fragments of 1235 bp and 611 bp respectively (figure 53 B red arrows). Additionally bands at approximately 3000 bp (pJET backbone) and approximately 250 bp (possibly an indication for a double ut by *Hind*III see figure 53 B) are also visible for the double digestion of the

frt_3´cbh construct. The double digestion of the frt_CAT construct also resulted in an unexpected band at around 900 bp (figure 51 lanes 1-6). This band could be caused by contaminations during the plasmid isolation. Finally the sequence of the frt_CAT and frt_3´cbh constructs were verified by sequence analysis (LGC genomic GmbH). The sequences obtained from LGC genomics matched the sequence of the *3´cbh* and *CAT* sequence in the plasmid pMS470_5´3´cbh_CAT (CD-Lab Graz strain collection #7420) and the *frt*-sites from the primer 180 and 182.

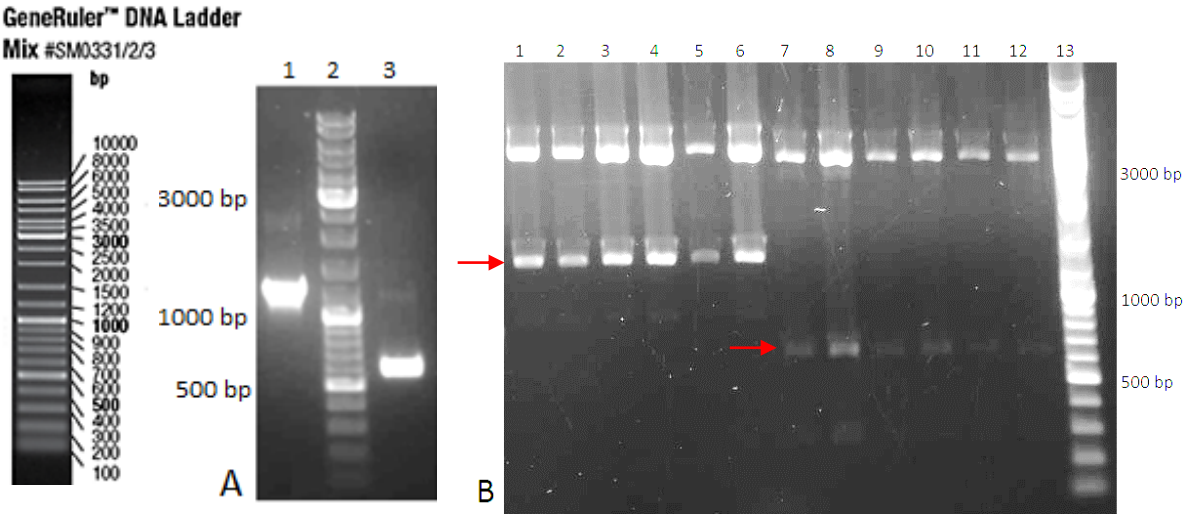

Figure 53: Cloning of the frt_CAT and frt_3´cbh fragments. *(A) Agarose gel (1%) of the PCR products using the primers 180 and 181 as well as 182 and 183 and the plasmid pMS470_5´3´cbh_CAT as template. lane 1: frt_CAT (expected size approximately 1200bp); lane 2: GeneRuler™ DNA Ladder Mix; lane 3: frt_3´cbh (expected size approximately 600 bp). (B) Agarose gel (1%) of the double digestions of the pJET_frt_CAT with XhoI, BamHI and pJET_frt_3´cbh with BamHI and HindIII. Lanes 1-6 pJET_frt_ (expected band sizes approximately 3000 bp and 1200 bp); lanes 7-12: pJET_frt_3´cbh (expected band sizes approximately 3000 bp and 600 bp); lane 13: GeneRuler™ DNA Ladder Mix. The frt_CAT and frt_3´cbh fragments are indicated by red arrows.*

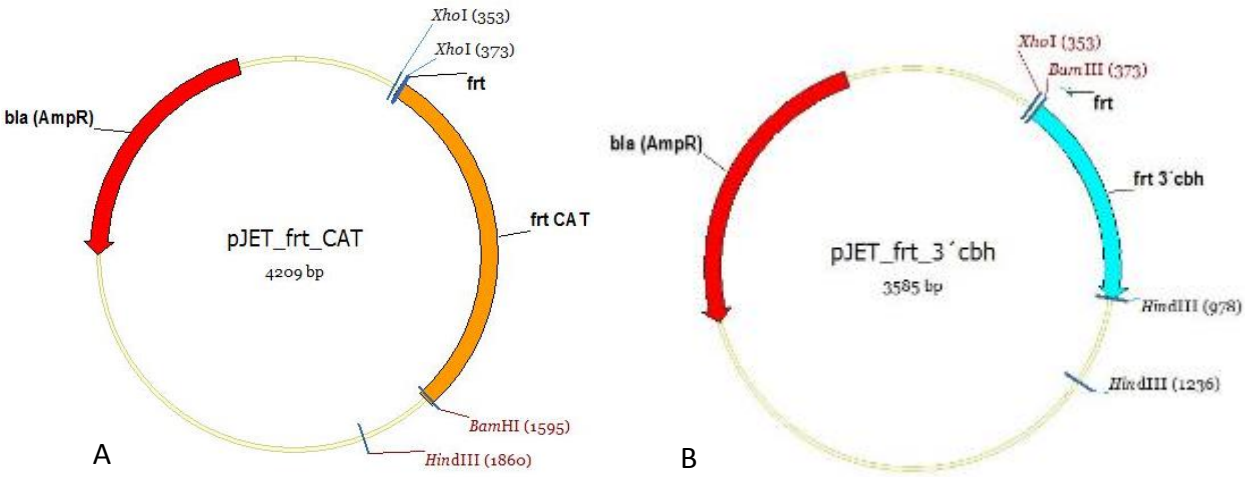

Figure 54: Plasmid maps of pJET_frt_CAT (A) and pJET_frt_3´cbh (B). *The positon of the resistance gene (bla) and the fragments frt_CAT (A) and frt_3´cbh (B) are shown. Additionally the position of the used restriction enzyme cleavage sites are indicated.*

In the next step the frt_CAT (from pJET_frt_CAT) and frt_3´cbh (from pJET_frt_3´cbh) were cloned together using the restriction sites *Bam*HI and *Hind*III, to generate the plasmid pJET_fcf3c (figure 55). This plasmid was used for transformation into electrocompetent *E. coli* XL1-Blue cells. 8 ampicillin resistant transformants were used for plasmid isolation and control digestion with *Xho*I and *Hind*III which resulted in the expected fragments of 1826 bp (frt_CAT_frt_3´cbh fragment) and 2700 bp (pJET backbone) which are shown in figure 56.

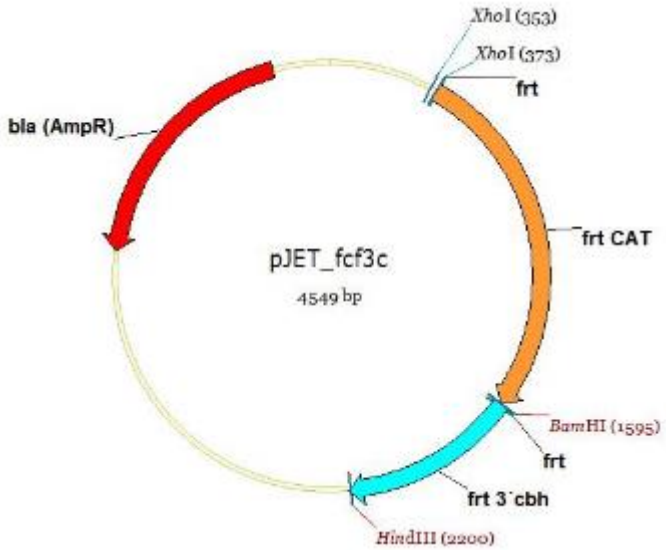

Figure 55: Plasmid maps of pJET_fcf3c. *The positon of the resistance gene (bla) and the fragments frt_CAT_frt_3´cbh is shown. Additionally the position of the used restriction enzyme cleavage sites are indicated.*

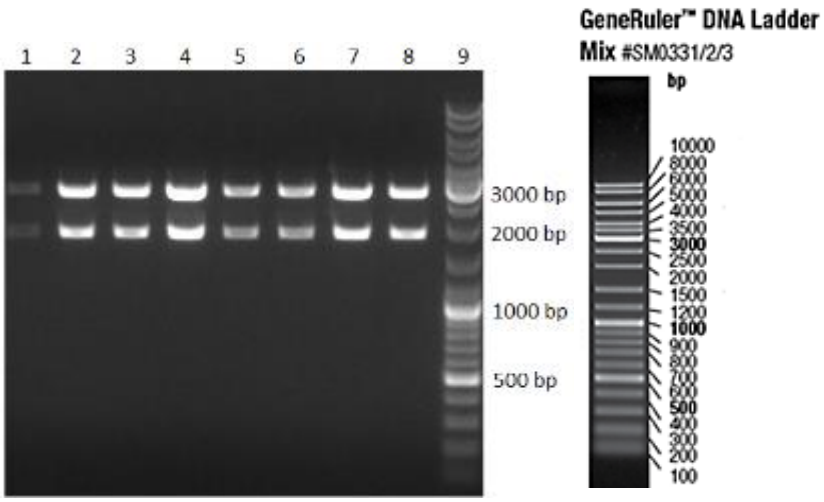

Figure 56: Cloning of the frt_CAT_frt_3´cbh fragment. *Agarose gel (1%) of the double digestion of the pJET_fcf3c using XhoI and HindIII. Lanes 1-8 pJET_fcf3c with XhoI, HindIII (expected band sizes of approximately 1826 bp and 2700 bp); lane 9: GeneRuler™ DNA Ladder Mix*.

Linkage of the 5´cbh efp celAocES constructs and the frt CAT frt 3´cbh construct to generate the 5´cbh efp celAocES frt CAT frt 3´cbh integration cassettes.

The 5´cbh_efp_celAocES constructs (with the different promoter modifications) were isolated from the respective plasmids pINT_term_5´cbh_efp_celAocES, pINT_term_5´cbh_efpshort1_celAocES and pINT_term_5´cbh_efpshort2_celAocES by double digestion with *Eco*RI and *Xho*I followed by agarose gel purification. The double digestion of pINT_term_5´cbh_efpshort1_celAocES was not successful because only a band at approximately 7300 bp is visible (figure 57). This corresponds to the size of the linear pINT_term_5´cbh_efpshort1_celAocES which suggests that only one of the restriction enzymes cleaved successfully. The digestion of the plasmid pINT_term_5´cbh_efp_celAocES resulted in the fragments with the expected sizes of 1944 bp (5´cbh_efp_celAocES) and 5410 bp (vector backbone, figure 57, lane 2). The digestion of the plasmid pINT_term_5´cbh_efpshort2_celAocES also resulted in fragments of the expected sizes of 1832 bp (5´cbh_efpshort2_celAocES) and 5410 bp (vector backbone, figure 57, lane 4). Additionally a band at about 3200 bp is visible. This band could represent non digested circular DNA which shows faster migration in the agarose gel due to supercoiling. The presence of circular DNA could be the result of the high DNA concentration used in this experiment. All further cloning steps were performed with the 5´cbh_efp_celAocES and the _5´cbh_efpshort2_celAocES fragments because the digestion of pINT_term_5´cbh_efpshort1_celAocES was not successful.

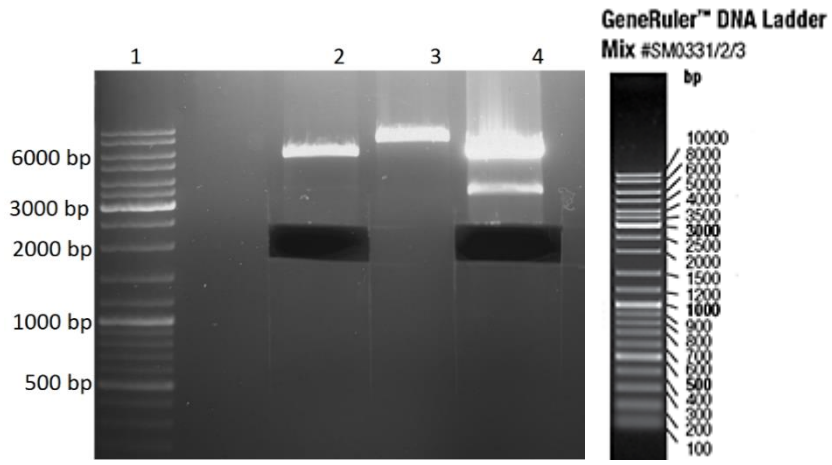

Figure 57: Isolation of the 5´cbh_efpcelAocES constructs. *Agarose gel (1%) of the double digestion (EcoRI and XhoI) of the plasmids pINT_term_5´cbh_efp_celAocES, pINT_term_5´cbh_efpshort1_celAocES and pINT_term_5´cbh_efpshort2_celAocES. Lane1: GeneRuler™ DNA Ladder Mix.; lane 2: pINT_term_5´cbh_efp_celAocES (expected band size 1944 bp and 5410 bp); lane 3: pINT_term_5´cbh_efpshort1_celAocES (expected band size 1891 bp and 5410 bp); lane 4: pINT_term_5´cbh_efpshort2_celAocES (expected band size 1832 bp and 5410 bp).*

Furthermore the frt_CAT_frt_3´cbh construct was also isolated from the plasmid pJET_fcf3c using the restriction enzymes *Xho*I and *Hind*III. Both, the 5´cbh_efp_celAocES and the 5´cbh_efpshort2_celAocES fragments were ligated to the frt_CAT_frt_3´cbh fragment in a 1:1 ratio and the resulting construct was amplified by Standard PCR using the primers 122 and 183 (see table 3). This PCR was performed using different volumes of the purified DNA from the ligation reaction (1µl of a 1:10 dilution, 1µl and 2µl) and resulted in the expected bands of 3657 bp or 3764 bp (figure 568, red arrows). These bands represent the 5´cbh_efp_celAocES_frt_CAT_frt_3´cbh and 5´cbh_efpshort2_celAocES_frt_CAT_frt_3´cbh fragments. Additionally unspecific bands at around 600 bp and 900 bp are visible. The intensity of these unspecific bands decreases with increasing template DNA concentration. This could be an indication for an inappropriate primer to DNA ratio which may result in non-specific amplification and primer-dimer formation.

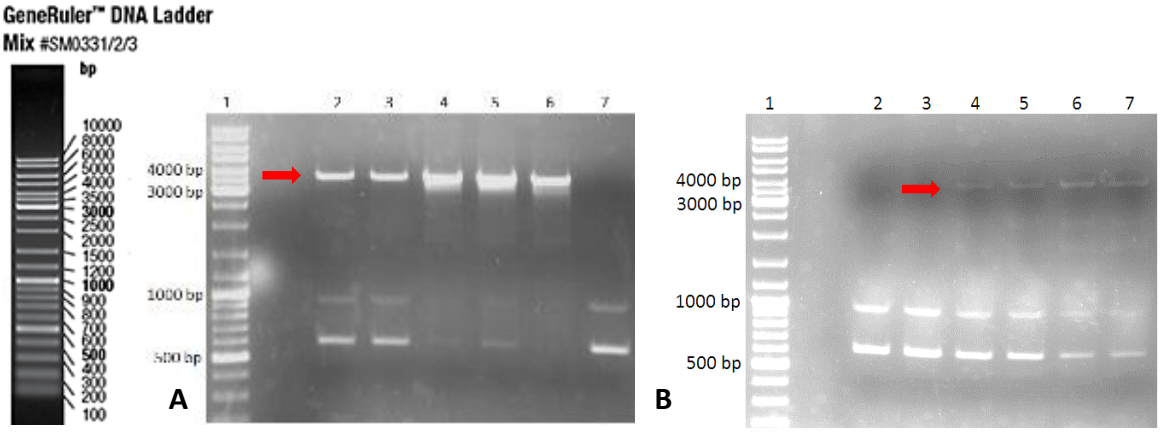

Figure 58: Amplification of the 5´cbh_efp_celAocES_frt_CAT_frt_3´cbh construct and 5´cbh_efpshort2_celAocES_frt_CAT_frt_3´cbh construct. *(A) Agarose gel (1%) of the PCR using the primer 122 and 183 and the product from the ligation reaction of the 5´cbh_efpshort2_celAocES construct from pINT_term_5´cbh_efpshort2_celAocES with the frt_CAT_frt_3´cbh as template. Lane 1: GeneRuler™ DNA Ladder Mix; lanes 2 and 3: 1µl of the 1:10 diluted ligation product (expected band size 3657 bp); lanes 4 and 5: 1 µl of the ligation product (expected band size 3657 bp); lanes 6 and 7: 2µl of the ligation product (expected band size 3657 bp). (B) Agarose gel (1%) of the PCR using the primers 5'cbh552_EcoRI_fwd, HindIII-EcoRI-3´cbh-rev and the product from the ligation reaction of the 5´cbh_efp_celAocES construct from the pINT_term_5´cbh_efp_celAocES with the frt_CAT_frt_3´cbh as template. 1: GeneRuler™ DNA Ladder Mix; lanes 2 and 3: 1µl of the 1:10 diluted ligation product (expected band size 3764 bp); lanes 4 and 5: 1 µl of the ligation product (expected band size 33764bp); lanes 6 and 7: 2µl of the ligation product (expected band size 3764 bp).*

The 5´cbh_efp_celAocES_frt_CAT_frt_3´cbh and 5´cbh_efpshort2_celAocES_frt_CAT_frt_3´cbh fragments generated by PCR were purified by agarose gel electrophoresis and cloned into the pJET1.2 blunt vector resulting in the plasmids pJET_5´cbh_efp_celAocES_fcf3c and pJET_5´cbh_efpshort2_celAocES_fcf3c (figure 59). These plasmids were transformed into electrocompetent *E. coli* XL1-Blue cells. Several resulting ampicillin resistant transformants were spotted onto LB-Amp-CMC plates to verify endoglucanase activity (figure 60 and 61, transformants used for plasmid isolation and control digestion are indicated by numbers).

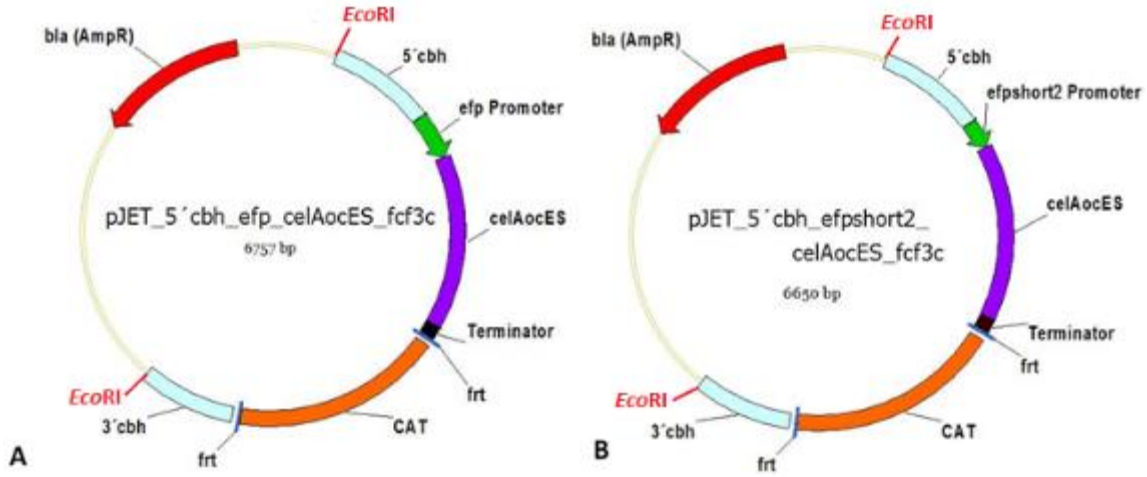

***Figure 59**: **Plasmid map of pJET_5´cbh_efp_celAocES_fcf3c (A) and pJET_5´cbh_efpshort2_celAocES_fcf3c (B).** The positon of the resistance gene (bla) and the integration cassettes (5´cbh_efp_celAocES_fc3c and 5´cbh_efpshort2_celAocES_fc3c) are shown. Additionally the position of the restriction enzyme cleavage sites used for the control digestion are indicated.*

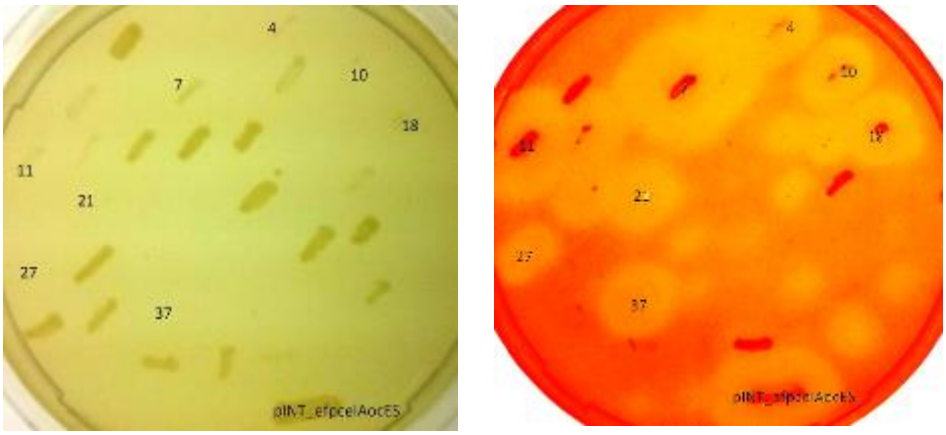

***Figure 60: Congo red-assay of the pJET_5´cbh_efpshort2_celAocES_fcf3c transformants.** Ampicillin resistant colonies of the transformation experiment using pJET_5´cbh_efpshort2_celAocES_fcf3c were spotted onto LB-Amp-CMC plates. The yellow halos indicate the endoglucanase activity. E. coli XL1-Blue [pINT_efpcelAocES] was used as endoglucanase positive control. Numbers indicate the further investigated colonies.*

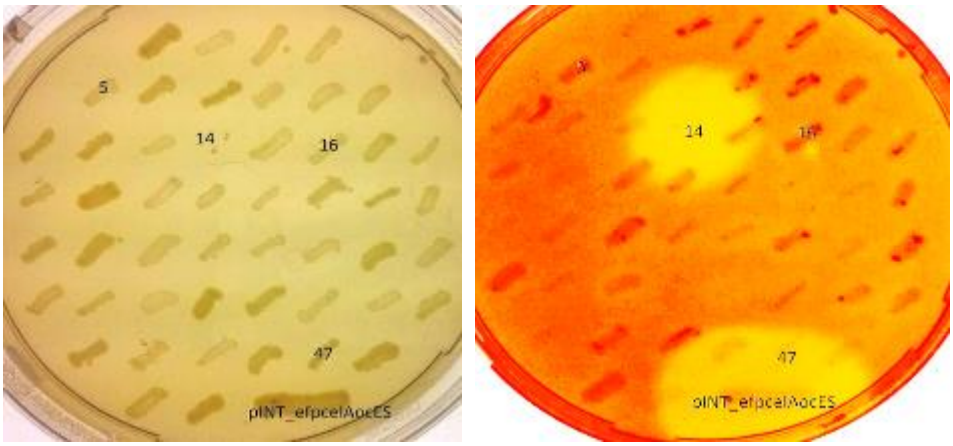

Figure 61: Congo red-assay of the pJET_5´cbh_efp_celAocES_fcf3c transformants. *Ampicillin resistant colonies of the transformation experiment using pJET_5´cbh_efp_celAocES_fcf3c spotted onto LB-Amp-CMC plates. The yellow halos indicate the endoglucanase activity. E. coli XL1-Blue [pINT_efpcelAocES] was used as endoglucanase positive control. Numbers indicate the further investigated colonies.*

The endoglucanase positive transformants were used for plasmid isolation and control digestion with *Eco*RI to verify the successful cloning of the 5´cbh_efp_celAocES_fcf3c and the 5´cbh_efpshort2_celAocES_fcf3c fragments into the pJET1.2 blunt (figure 62). The expected sizes for the fragments resulting from the digestion of the pJET_5´cbh_efp_celAocES_fcf3c are 3770 bp (5´cbh_efp_celAocES_fcf3c fragment) and 2986 bp (pJET backbone, figure 62 lanes 10-13) while the expected sizes resulting from the digestion of the pJET_5´cbh_efpshort2_celAocES_fcf3c are 3663 bp (5´cbh_efpshort2_celAocES_fcf3c fragment) and again 2986 bp (pJET backbone, figure 62 lanes 2-9). The plasmid pJET_5´cbh_efpshort2_celAocES_fcf3c #4 (figure 62, lane 2) and pJET_5´cbh_efp_celAocES_fcf3c #14 (figure 62, lane 11) showed the expected band profile and were used for further verification by sequence analysis (LGC genomics GmbH). The accuracy of the produced fragment DNA (5´cbh_efp_celAocES_fcf3c and 5´cbh_efpshort2_celAocES_fcf3c) were confirmed by alignment of the sequence obtained from LGC genomics with the reference sequences of the plasmids pJET_5´cbh_efp_celAocES_fcf3c and pJET_5´cbhshort2_efp_celAocES_fcf3c (IMBT strain collection #7418 and #7419). Subsequently these plasmids were used for the generation of dsDNA required for the integration experiments into *L. plantarum* CD033 and 3NSH.

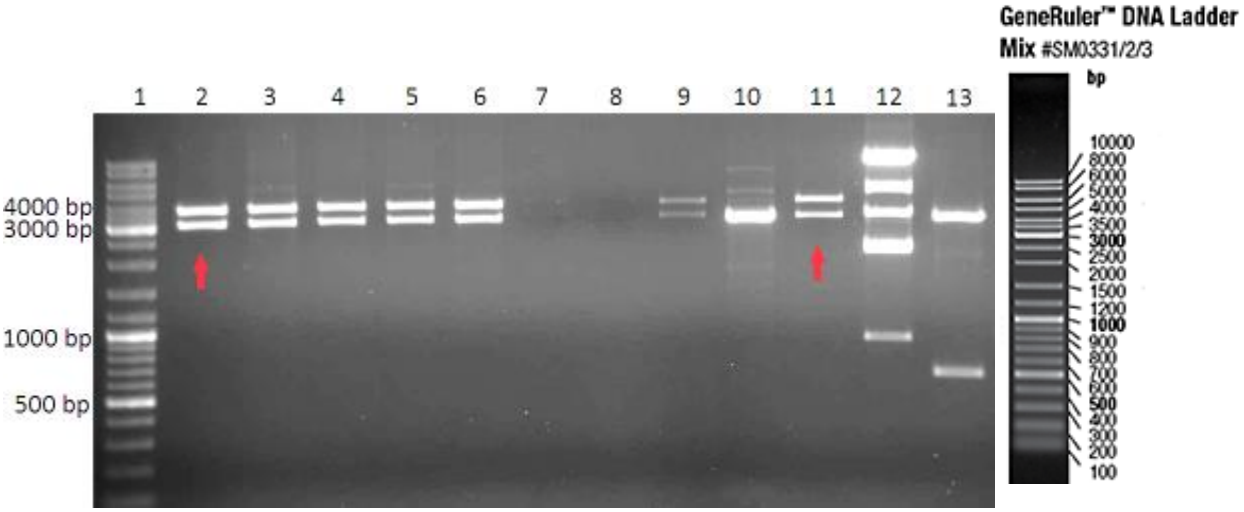

Figure 62: Control digestion of plasmids isolated from the endoglucanase positive transformants. *Agarose gel (1%) of the plasmids pJET_5´cbh_efp_celAocES_fcf3c and pJET_5´cbh_efpshort2_celAocES_fcf3c after EcoRI digestion. Lane 1: GeneRuler™ DNA Ladder Mix; lanes 2-9: pJET_5´cbh_efpshort2_celAocES_fcf3c number 4, 7, 10, 11, 18, 21, 27, 37 (expected band size 3663 bp and 2986 bp); lanes 10-13: pJET_5´cbh_efp_celAocES_fcf3c number 5, 14, 16, 47 (expected band size 3770 bp and 2986 bp). The constructs used for sequence analysis are marked with red arrows.*

5.8 Generation of linear dsDNA of the Flp/*frt* based integration cassette and transformation into *L. plantarum*

Because all previous integration experiments using ssDNA were not successful, the integration experiments with the Flp/*frt* based integration cassettes (5´cbh_efp_celAocES_fcf3c and 5´cbh_efpshort2_celAocES_fcf3c) were only performed using linear dsDNA.

The linear dsDNA was obtained by Standard PCR using pJET_5´cbh_efpshort2_celAocES_fcf3c #4 and pJET_5´cbh_efp_celAocES_fcf3c #14 as templates (described in part 4.8, primer 138 and 139 see table 6). In figure 63 the PCR products are shown (expected band size of 3770 bp for 5´cbh_efp_celAocES_fcf3c and 3663 bp for 5´cbh_efpshort2_celAocES_fcf3c).

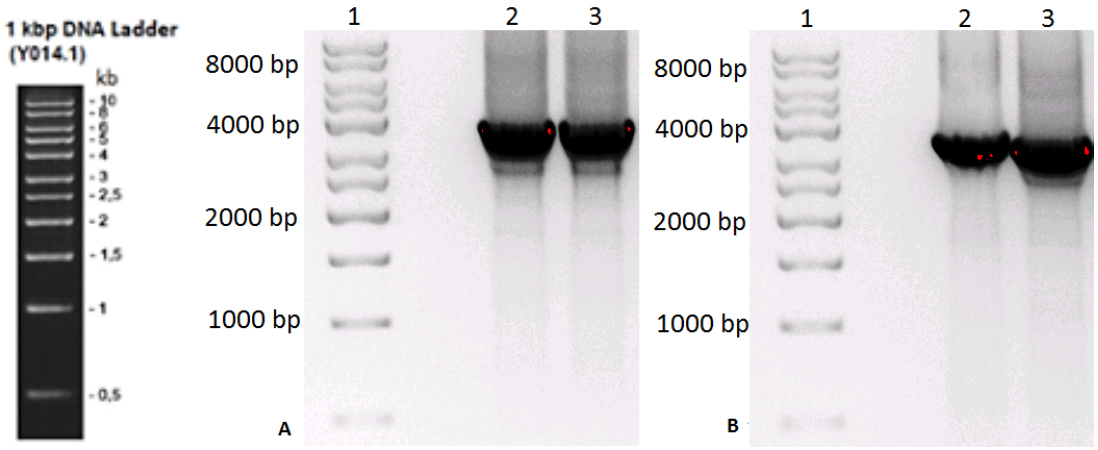

Figure 63: Amplification of the Flp/frt based integration cassette. *(A) Agarose gel (1%) of the PCR products using the primers 138 and 139 with the plasmid pJET_5´cbh_efp_celAocES_fcf3c #14 as template. Lane 1: 1 kbp DNA ladder (Carl Roth); lanes 2 and 3: PCR product (expected fragment size of 3770 bp); (B) Agarose gel (1%) of the PCR products using the primers 5'cbh_plantplant_fwd and 3'cbh_linear_rev with the plasmid pJET_5´cbh_efpshort2_celAocES_fcf3c #4 as template. Lane 1: 1 kbp DNA ladder (Carl Roth); lanes 2 and 3: PCR product (expected fragment size of 3663 bp).*

Next, 5 µg and 10 µg of the generated linear dsDNA of each construct (5´cbh_efp_celAocES_fcf3c and for 5´cbh_efpshort2_celAocES_fcf3c) were transformed into *L. plantarum* CD033 and 3NSH. The transformation experiment using the strain *L. plantarum* CD033 did not result in any chloramphenicol resistant colonies. However, the transformation experiment using the strain *L. plantarum* 3NSH resulted in 2 putative chloramphenicol resistant colonies. To verify the chloramphenicol resistance, the colonies were streaked onto MRS-CM plates. Further the cells were assessed by microscopic examination to confirm the rod-shaped morphology of the bacterium. Only a single chloramphenicol resistant colony (3NSH A5-1) was obtained. This colony was streaked onto a MRS-CM-CMC plate to verify endoglucanase activity. The Congo red- assay resulted in the formation of a yellow halo that indicates the desired endoglucanase activity (figure 64). This colony resulted from the integration of the 5´cbh_efp_celAocES_fcf3c DNA fragment.

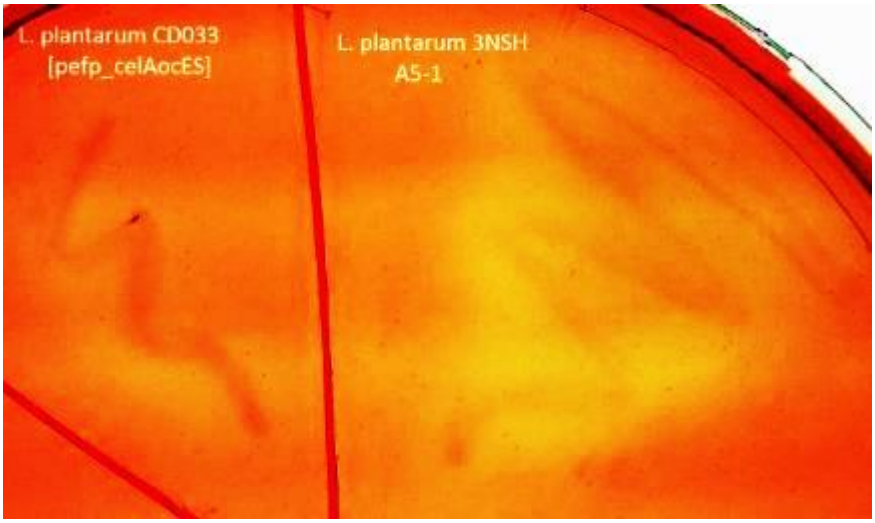

Figure 64: Congo red-assay of the transformant 3NSH A5-1*. The chloramphenicol resistant colony of the transformation experiment using linear dsDNA of the 5´cbh_efp_celAocES_fcf3c fragment was plated onto MRS-CM-CMC plates. The yellow halos indicate the endoglucanase activity. L. plantarum CD033 [pefp_celAocES] was used as endoglucanase positive control.*

In order to verify the correct integration of the 5´cbh_efp_celAocES_fcf3c fragment into the genome, the genomic DNA of the transformant 3NSH A5-1 was isolated (DNeasy Blood&Tissue Kit, Qiagen) and investigated by PCR using different primer combinations. The different primer combinations used are shown in figure 65 (primer details see table 5) and the expected fragment sizes are summarized in table 23.

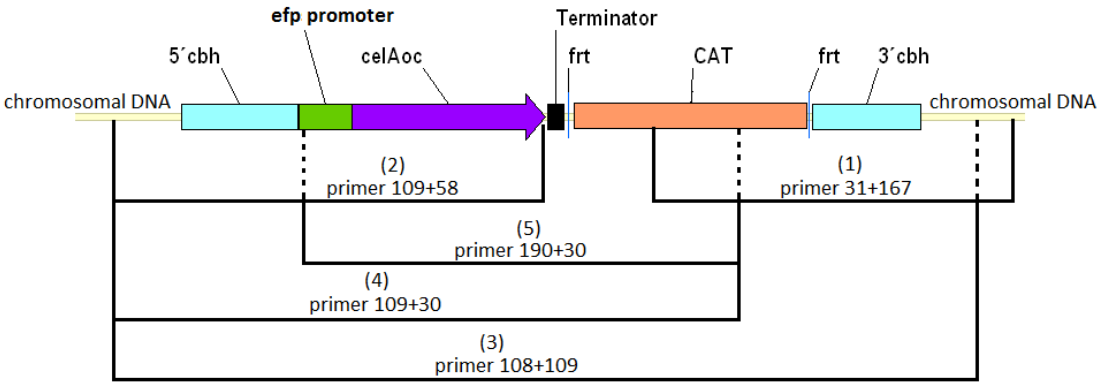

Figure 65: Schematic representation of the 5´cbh_efp_celAocES_fcf3c cassette after integration into the host genome. *The primer combinations used for integration confirmation are indicated by lines and the abbreviations are shown in brackets.*

Table 23: Fragment sizes of primer combination

Primer combination	Fragment size (bp)
(1) 31+167	~1500
(2) 109+58	~2200
(3) 108+109	~4000
(4) 30+109	~3000
(5) 30+190	~2100

The primer combination 1 (figure 66 lane 2) resulted in the expected fragment of 1500 bp. The primer combination 2 (figure 66 lane 5) amplified two fragments. The fragment at around 2200 bp resembles the expected fragment, while the band at around 6000-7000 bp could be the result of two 5´cbh_efp_celAocES_fcf3c fragments integrated in consecutive fashion. The primers 108 and 109 (primer combination 3, figure 66 lane 8) generated the desired fragment of 4000 bp. Additionally a faint band at about 1500 bp and band of higher intensity at about 8000 bp are visible. The band at 1500 bp could be an indication for additional wild type *cbh* genes present in the genome of the used *L. plantarum* strain. Another possible explanation could be the presence of wild type *L. plantarum* during the genomic DNA isolation. The 8000 bp fragment generated by the primer combination 3 suggests a consecutive integration of two 5´cbh_efp_celAocES_fcf3c fragments. Beside the expected band at 3000 bp the primer combination 4 (figure 66 lane 11) shows two additional higher bands (at about 7000 bp and higher than 10000 bp) that again could indicate consecutive integration of two and/or multiple 5´cbh_efp_celAocES_fcf3c fragments. Finally the band generated by the primer combination 5 (figure 66 lane 15) shows the expected size of 2100 bp but appears a little smeared like the bands in lane 2 and 9. Despite using the same amount of template DNA in all reactions these bands show a very high intensity. This could indicate multiple integration of the 5´cbh_efp_celAocES_fcf3c fragment. To verify that the used primer combinations did not amplify any parts of the genome from the wild type *L. plantarum* strain 3NSH, genomic DNA was isolated and used as template for PCR with the different primer combinations (figure 66 lanes 3, 6, 9, 12 and 16). The related PCR mastermixes (all components except template DNA) were also investigated but showed no noteworthy bands (figure 66 lanes 4, 7, 10, 13 and 17).

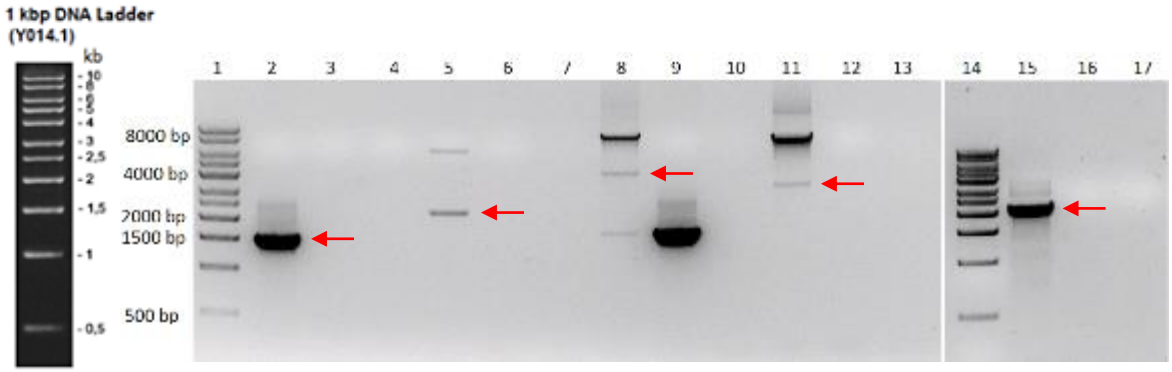

Figure 66: Verification of the integration of 5´cbh_efp_celAocES_fcf3c. *Agarose gel (1%) of PCR using different primer combinations and the genomic DNA of the transformant 3NSH A5-1 to verify the integration of the Flp/frt based integration cassette (5´cbh_efp_celAocES_fcf3c). Lanes 1 and 14: 1 kbp DNA ladder (Carl Roth); lane 2: primer combination 1 (31+167, expected size 1500 bp); lane 5: primer combination 2 (109+58, expected size 2200 bp); lane 8: primer combination 3 (108+109, expected size 4000 bp); lane 11: primer combination 4 (30+109, expected size 3000 bp); lane 15: primer combination 5 (30+190, expected size 2100 bp); lanes 3, 6, 9, 12 and 16: PCR of the respective primer combinations using genomic DNA isolated from the wild type L. plantarum; lanes 4, 7, 10, 13 and 17: respective PCR mastermixes. The expected fragments are indicated by red arrows.*

To determine whether the integration is stable over a longer period of time, a stability assay was performed. Therefore, a liquid culture with the transformant 3NSH A5-1 (without antibiotic) was inoculated for 28 days. Everyday a determination of colony-forming units (CFU) was done by plating 100 µl of a 10^{-4} to 10^{-6} dilution onto MRS and MRS-CM plates. In figure 67 the diagram of the integration stability assay is shown ((A) in percentage and (B) the CFU/ml).

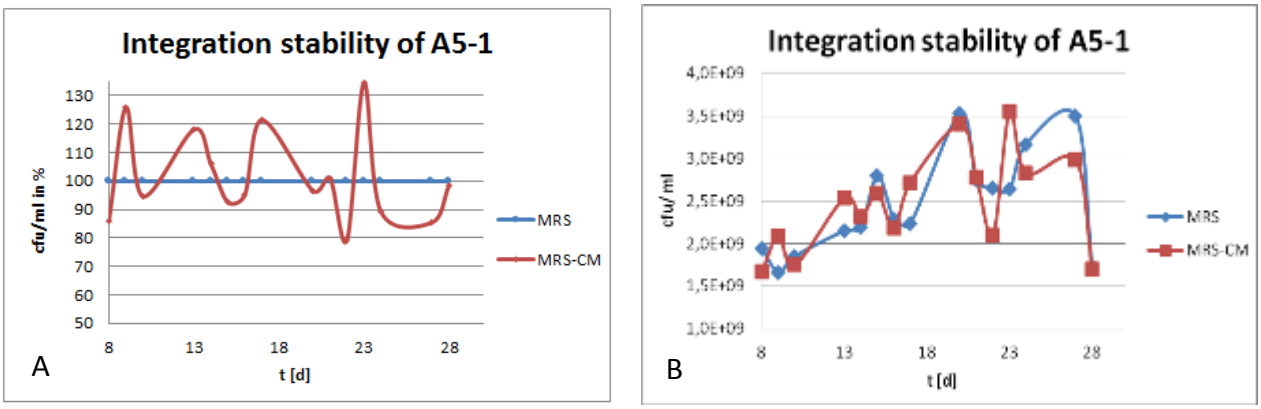

Figure 67: Diagrams of the integration stability assay of the A5-1 integrant. *(A) Stability of genome integration in percentage and in the CFU/ml (B).*

The numbers of bacteria on MRS-CM remain in the range of 10^9 CFU/ml and are similar to the numbers of bacteria on MRS without antibiotics. This indicates that the integration is stable for at least 28 days.

To confirm the integration stability on the molecular level, some colonies of the transformant 3NSH A5-1 were picked from the MRS-CM plates after 20 days of inoculation and used for genomic DNA isolation. Afterwards the genomic DNA of the colonies from day 20 as well as the genomic DNA isolated from the original 3NSH A5-1 (day 0) were used as templates for PCR with the previously used primer combinations (figure 65, primer details see table 5) listed in table 23. The resulting agarose gel pictures are shown in figure 68-70. The majority of the investigated colonies showed the same band profile as transformant 3NSH A5-1 at the beginning of the stability assay, suggesting that the integration is stable for at least 20 days.

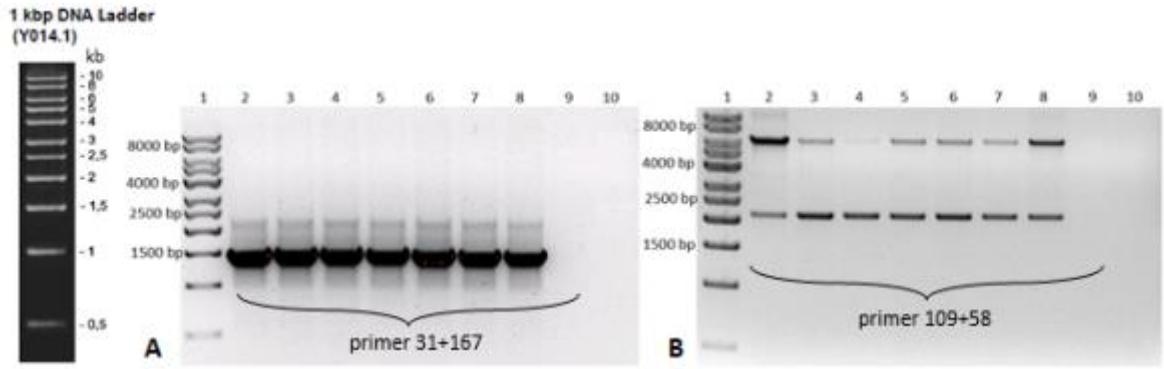

Figure 68: Verification of the integration stability using primer combination 1 and 2. *Agarose gel (1%) of PCR using different primer combinations and the genomic DNA of the colonies from transformant 3NSH A5-1 after 20 days of inoculation compared to the genomic DNA of the original transformant 3NSH A5-1 (day 0) as template. (A) primer combination 31+167 (expected size 1500 bp). Lane 1: 1 kbp DNA ladder (Carl Roth); lane 2: genomic DNA isolated from the original transformant 3NSH A5-1 (day 0); lanes 3-8: genomic DNA isolated from colonies of the transformant 3NSH A5-1 after 20 days of inoculation; lane 9: genomic DNA of the L. plantarum wild type 3NSH; lane 10: PCR mastermix. (B) primer combination 109+58 (expected size 2200 bp) Lane 1: 1 kbp DNA ladder (Carl Roth); lane 2: genomic DNA isolated from the original transformant 3NSH A5-1 (day 0); lanes 3-8: genomic DNA isolated from transformants 3NSH A5-1 after 20 days of inoculation; lane 9: genomic DNA of the L. plantarum wild type 3NSH; lane 10 PCR mastermix.*

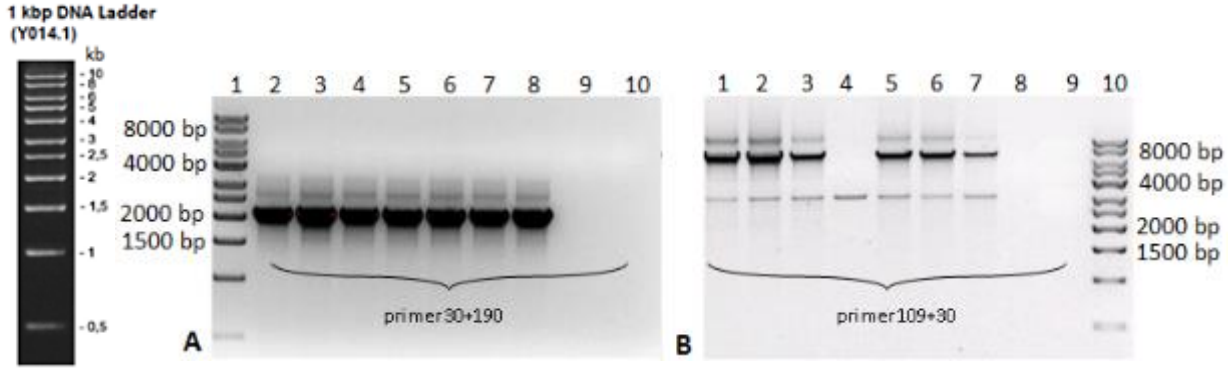

Figure 69: Verification of the integration stability using primer combination 4 and 5. *Agarose gel (1%) of PCR using different primer combinations and the genomic DNA of the colonies from transformant 3NSH A5-1 after 20 days of inoculation compared to the genomic DNA of the original transformant 3NSH A5-1 (day 0) as template. (A) primer combination 5 (primers 30+190, expected size 2100 bp). Lane 1: 1 kbp DNA ladder (Carl Roth); lane 2: genomic DNA isolated from the original transformant 3NSH A5-1 (day 0); lanes 3-8: genomic DNA isolated from transformants 3NSH A5-1 after 20 days of inoculation; lane 9: genomic DNA of the L. plantarum wild type 3NSH; lane 10: PCR mastermix. (B) primer combination 4 (primers 109+30, expected size 3000 bp) Lane 1: genomic DNA isolated from the original transformant 3NSH A5-1 (day 0); lanes 2-7: genomic DNA isolated from transformants 3NSH A5-1 after 20 days of inoculation; lane 8: genomic DNA of the L. plantarum wild type 3NSH; lane 9: PCR mastermix; Lane 10: 1 kbp DNA ladder (Carl Roth).*

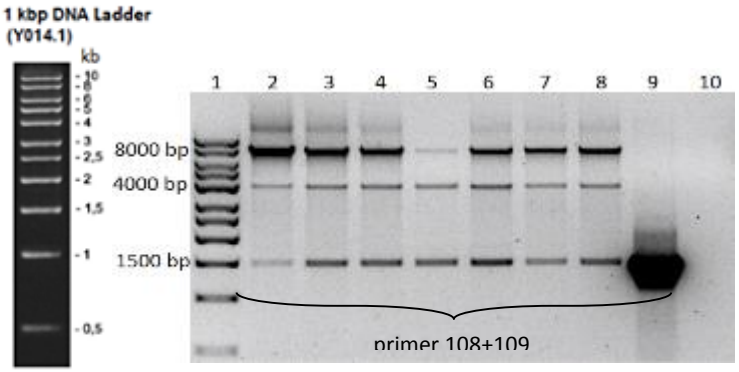

Figure 70: Verification of the integration stability using primer combination 3. *Agarose gel (1%) of PCR using different primer combinations and the genomic DNA of the transformant 3NSH A5-1 after 20 days of inoculation compared to the original transformant 3NSH A5-1 (day 0). Primer combination 3 (primers 109+108, expected size 4000 bp). Lane 1: 1 kbp DNA ladder (Carl Roth); lane 2: genomic DNA isolated from the original transformant 3NSH A5-1 (day 0); lanes 3-8: genomic DNA isolated from transformants 3NSH A5-1 after 20 days of inoculation; lane 9: genomic DNA of the L. plantarum wild type 3NSH; lane 10: PCR mastermix.*

To ultimately confirm the correct integration of the Flp/*frt* based integration cassette (5´cbh_efp_celAocES_fcf3c) into the genome of *L. plantarum* 3NSH, overlapping parts resembling the whole cassette were amplified using PCR with several primer combinations (summarized in figure 71).

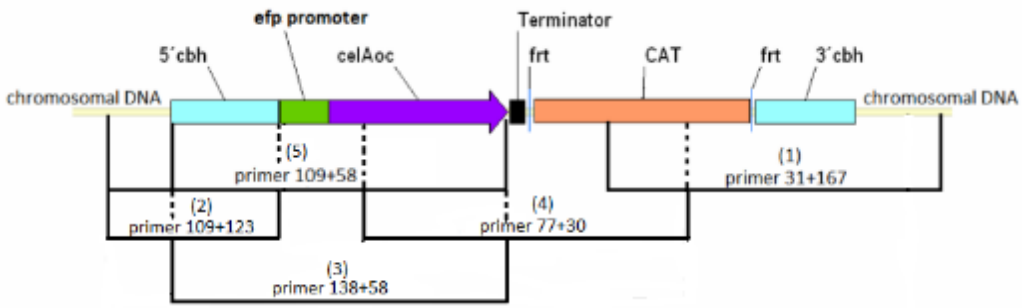

Figure 71: Schematic representation of the 5´cbh_efp_celAocES_fcf3c cassette for sequence analysis. *The primer combinations used for sequence analysis are indicated by lines and the abbreviations are shown in brackets.*

Table 24: fragment sizes of primer combination

Primer combination	Fragment size (bp)
(1) 31+167	~1500
(2) 109+123	~900
(3) 138+58	~1900
(4) 77+30	~1900
(5) 109+58	~2200

All used primer combinations resulted in fragments of the expected size which are summarized in figure 71 and in table 24 (primer details see table 5, agarose gel see figure 72).

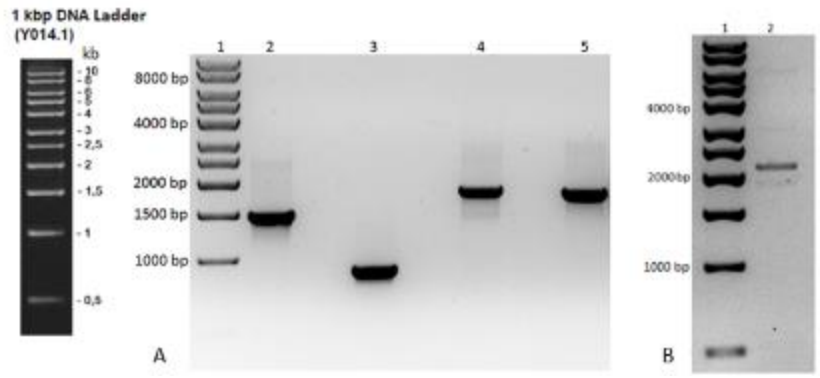

Figure 72: PCR fragments used for sequence analysis of the integrated 5´cbh_efp_celAocES_fcf3c cassette. *Agarose gel (1%) of PCR using different primer combinations and the genomic DNA of the original transformant 3NSH A5-1 (day 0) as template. (A): Lane 1: 1 kbp DNA ladder (Carl Roth); lane 2: primer combination 31+167 (expected size 1500 bp); lane 3: primer combination 109+123 expected size (900 bp); lane 4: primer combination 138+58 (expected size 1900 bp); lane 5: primer combination 77+30 (expected size 1900 bp). (B): lane 1: 1 kbp DNA ladder (Carl Roth); lane 2: primer combination 109+58 (expected size 2200 bp).*

The PCR products were purified using the GeneJET PCR Purification Kit (Thermo Scientific) as described in part 4.1.5 and used for sequence analysis (Eurofins genomics).

The sequence analysis and subsequent alignment with corresponding reference sequences confirmed the sequence identity and integration locus (figure 73). The green bar in figure 73 symbolizes sequences without mismatches. The yellow bar denotes sequences with some mismatches.

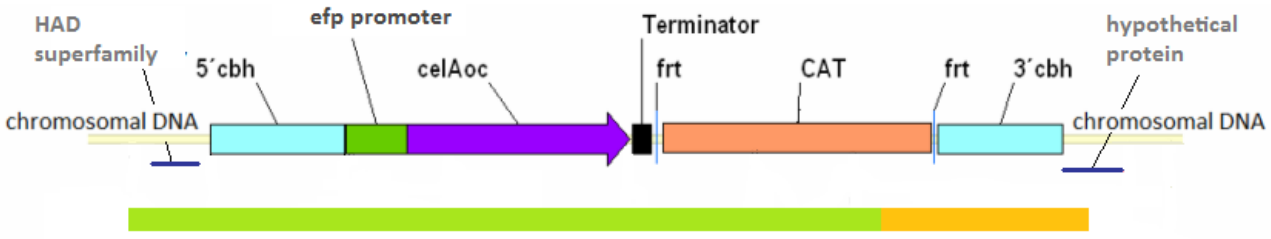

Figure 73: Scheme of the sequence alignment. *The green bar represents sequences without mismatches, the yellow bar represent sequences with some mismatches.*

6 Discussion

6.1 Heterologous expression and purification of the His_6-tagged SSB and RecA

In order to investigate possible optimizations of integration of genetic material into the *L. plantarum* genome, one aim of this thesis was to test the integration of linear ssDNA sequences. Because of its instability, naturally occurring ssDNA is bound and stabilized by several ssDNA binding proteins [39]. One of the most common ssDNA binding proteins is the single-stranded DNA binding protein (SSB) which is well known to protect and stabilize single stranded sequences [10-12, 17]**.** Loading of ssDNA with DNA binding proteins like SSB and RecA prior to the transformation into *L. plantarum* could stabilize and protect the ssDNA from degradation by host enzymes and therefore increase the integration efficiency. To heterologously express SSBnHis and RecAnHis, the strains *E. coli* BL21DE3 [pMS470nHis_ssb], *E. coli* BL21DE3 [pET28b_ssb] and *E. coli* BL21DE3 [pMS470nHis_recA], which carry plasmids coding the genes of SSB and RecA isolated from *L. plantarum* CD033, were used. The first preliminary investigation of the expression strains by Western blot showed that all strains heterologously expressed the respective proteins (figures 38, 39 and 40). The Westernblot of the *E. coli* BL21DE3 [pMS470nHis_ssb] (figure 38 B, lane 1 and 2) only showed a band in the insoluble fraction. Comparison with the respective lanes of the SDS gel (figure 38 A, lane 1 and 2) reveal that a similar band of the same size is also present in the soluble fraction. The loss of this band in the Western blot could be caused by a transfer problem like an air bubble between gel and membrane. Estimating the size of the investigated proteins using the indicated SDS PAGE standards leaded to an unexpected molecular weight which did not match the theoretical sizes of 23.8 kDa for SSBnHis and 42.9 kDa for RecAnHis (as calculated by the ExPASy Compute pI/Mw tool). Instead SSBnHis and RecAnHis showed a shift of approximately 8-10 kDa (figure 38 A and figure 39 and 40). A possible explanation of the unexpected molecular weight shift could be posttranslational modifications by the used *E. coli* BL21DE3 strain. It has previously been reported that this *E. coli* strain is able to introduce non-enzymatic posttranslational modifications into

recombinant proteins [37]. Furthermore SSB [40, 41] and RecA [42] have been reported to be targets for posttranslational modifications. Depending on the frequency of posttranslational modifications, like phosphorylation or glycosylation, it is unlikely that these are the sole reason for the observed molecular weight shift. However it is possible that such modifications contribute to the observed shift. Furthermore it is possible that the His_6-tag [43] or inappropriate denaturation of the proteins contributes to the altered electrophoretic mobility. Another reason for the increased molecular weight of RecA is the use of a primer that removed the stop codon of the RecA. Because of this mistake the used RecAnHis has 7 additional amino acids at its C-terminus (KLGCFGG) which increases its theoretical size to 43.5 kDa. However the identity of the heterologously expressed proteins has been confirmed by sequencing of the cDNA encoding SSBnHis and RecAnHis. Ultimately, the identity of both recombinant proteins could be further confirmed by mass spectroscopy. This analysis could also be used to identify possible posttranslational modifications that could contribute to the observed divergence of the theoretical and the apparent molecular weight. The recombinant SSBnHis and RecAnHis also showed different sizes depending on the used SDS buffer system. While SDS-PAGE using the MES buffer (pH 7.3) resulted in an approximated size of 32 kDa for SSBnHis (figure 38 A) and 50 kDa for RecA (figure 39 and 40), the Tris-glycin buffer (pH 8.6) resulted in an approximate molecular weight of 30 kDa for SSBnHis (figure 41) and 55 kDa for RecAnHis (figure 42). Depending on the pH of the buffer, the mobility of the proteins is different which could explain the observed difference in the molecular weight between the two buffer systems.

The purification of the heterologously expressed SSBnHis and RecAnHis using a Ni-NTA sepharose column resulted in an almost pure protein solutions. The majority of the His_6-tagged SSB (figure 41) and RecA (figure 42) were present in the elution fractions, while only a small portion remained in the insoluble fraction. Also only a small portion of the heterologously expressed proteins were present in the flow through (figure 41 lane 3 and figure 42 lane 2). This indicates that the initial protein concentration exceeded the column capacity. Therefore a possibility to increase the protein yield would be to use a column with higher volume or to apply the soluble fraction on multiple columns. Increasing the imidazole concentration in the Wash Buffer increased the purity of the elution fractions. However it was not possible to acquire completely pure proteins. A possibility to further increase the purity of the acquired heterologously expressed SSBnHis and RecAnHis would

be to use a second purification method. One such method would be size exclusion chromatography (SEC). This method separates the proteins based on their molecular size and shape as opposed to the highly specific interactions that are the basis of the used Ni-NTA sepharose column purification. Using a second purification method that is based on a different purification principle could remove impurities that remain after the initial purification step.

6.2 Generation of linear ssDNA of the 5´3´cbh_CAT cassette.

Simon & Moore et al. reported that integration of ssDNA is more efficient compared to dsDNA in *S. cerevisiae* [43] and it has also been shown that the use of ssDNA reduced the occurrence of non-homologous DNA integration by 100 fold in *Chlamydomonas reinhardtii* [44]. Therefore the use of ssDNA could also be a promising strategy to integrate DNA into the genome of *L. plantarum*. To generate ssDNA, asymmetric PCR using a 100 fold excess of either the forward or the reverse primer and the plasmid pMS470_5´3´cbh_CAT as template have been performed.

During the first approach to generate ssDNA the wrong temperature profile A has been used accidentally. The PCR has been performed using a Phusion polymerase with a temperature profile optimized for the DreamTaq polymerase. This PCR resulted in a fragment that matched the theoretical size (~2400 bp) of the desired DNA fragment. Additionally another fragment at approximately 5000 bp was generated (figure 43). This additional band could be caused by the inappropriate temperature profile used in this experiment. Another mistake during the approach using the temperature profile A was that the concentration of the template DNA has not been determined and documented appropriately. Due to the unknown template DNA concentration it is possible that a very unfavourable primer to DNA ratio has been used, which could lead to reduced amplification rate or smeared bands. While the approach using the temperature profile A resulted in the same band pattern with either the forward or reverse primer in 100 fold excess, the approach using the temperature profile B resulted in different patterns depending on the primer that has been used in excess. PCR using the forward primer in excess generated

several unspecific bands (figure 44 lanes 2-6). The same unspecific bands also appeared in the mastermix without template DNA. It is possible that the resulting bands are caused by primer dimer formation. Compared to the forward primer, excess of the reverse primer resulted in one band that matched the theoretical size of the desired DNA fragment. While evaluating the size of presumable ssDNA, it has to be considered that the electrophoretic mobility of ssDNA differs from dsDNA. Large ssDNA fragments are reported to have a decreased electrophoretic mobility compared to dsDNA [38].

The data of the PCR using the temperature profile B suggests that the 100:1 ratio of the reverse to forward primer is more suitable for the asymmetric PCR. On the other hand a recent report suggests a 20:1 ratio of forward to reverse primer for optimal ssDNA generation. However these experiments were only performed with excess of the forward primer [45]. It is possible that the primer ratio as well as the primer used in excess is primer dependent. Due to the low number of experiments the generation of ssDNA requires further optimization in regard to the primer ratio and primer to DNA ratio. Furthermore it is not clear if the DNA generated by the asymmetric PCR indeed is ssDNA. A possibility to distinguish between ssDNA and dsDNA would be the use of SYBR green which shows a stronger fluorescence when bound to dsDNA compared to ssDNA [46]. Another strategy to distinguish ssDNA from dsDNA would be to take advantage of the different electrophoretic mobility's of the two DNA species. Lim et al. showed that ssDNA migrates significantly faster in a 6 M urea PAGE which can be used to distinguish ssDNA from dsDNA [46].

6.3 Gel shift assay using ssDNA of the 5´3´cbh_CAT cassette

In order to protect and stabilize the ssDNA during the integration, the heterologously expressed DNA binding proteins have to be loaded onto the ssDNA. Therefore the in vitro ssDNA binding capability of SSBnHis and RecAnHis were investigated using a gel shift assay. In this assay the recombinant DNA binding proteins were incubated with ssDNA generated by asymmetric PCR using the temperature profile B. The Gel shift assay using the recombinant SSBnHis showed a quantity independent shift of the presumable ssDNA band at approximately 2400 bp (figure 45 lane 3, 4, 10 and 11). However a "cloud" that most

likely represents the protein/DNA complexes, showed an increased shift with increased SSBnHis. Previous gel shift experiments using SSB resulted in a concentration dependent shift without any “cloud” like bands (data not shown). Therefore it is important to note that the presented data only represents a single experiment and that the reproducibility is questionable. The addition of SSBnHis to dsDNA also resulted in a shift of the DNA band (figure 46 lane 2 and 3). This shift increased with increasing amount of SSBnHis, which indicates that the dsDNA was not saturated by 2 µg SSBnHis.

The addition of RecAnHis to the presumable ssDNA as well as dsDNA resulted in a concentration dependent vanishing of the DNA band while a significant amount of DNA was stuck in the slot of the agarose gel (figure 45 lanes 5, 6; 12 and 13; figure 45 lanes 4 and 5). This could be caused by the filament formation of RecAnHis around the DNA. Previous gel shift experiments using RecA also resulted in a vanishing of the DNA band while not showing the DNA stuck in the slot (data not shown). A possible explanation for the decreasing band intensities could be the formation of many different DNA/protein complexes with different degrees of saturation. These different complexes would migrate slower but could be below the detection limit of the ethidium bromide which would result in the observed decreased intensity of the DNA band. It is important to note that due to the divergences between the gel shift assays of RecA, the reproducibility of the experiments is questionable.

To confirm the specificity of the DNA/protein interactions the gel shift assay was also performed using a non DNA binding protein. For this purpose the protein alcohol dehydrogenase (ADH) isolated from *Ralstonia eutropha* H16 was used. Incubating ADH with ssDNA resulted in no significant change of the DNA band indicating no DNA/protein complex formation (figure 45 lanes 7, 8, 14 and 15). However, low intensity “clouds” in the range of 4000 bp – 8000 bp are visible. It is possible that the ADH protein preparation still contained DNA binding proteins that remained after the ADHnHis purification. Such impurities could cause unspecific DNA/protein complexes that result in the formation of the observed low intensity “clouds”. Due to a mistake during the sample application no assessment of the DNA/protein interactions of ADH with dsDNA can be made.

6.4 DNA/protein loading and transformation into *L. plantarum*

The next step was to investigate the transformation and integration of linear ssDNA protected by SSBnHis and RecAnHis compared to linear ssDNA alone and to linear dsDNA. Unfortunately the performed transformation and integration experiments using linear ssDNA of the short linear integration cassette (5´3´cbh_CAT) did not result in any chloramphenicol resistant transformants regardless of the presence of DNA binding proteins. A possible explanation for the failure of the ssDNA integration experiments could be the quality of the used ssDNA. During the generation of the ssDNA, a wrong temperature profile has been used accidentally. Because of this mistake it cannot be assured that the PCR resulted in the desired product. Additionally the template concentration used to generate the ssDNA was not determined and documented appropriately. Therefore the DNA/primer ratio of this experiment is not documented. It is possible that a very unfavourable DNA/primer ratio was used for the generation of the ssDNA which could lead to unspecific products. Because of the mistakes that happened during the ssDNA generation it cannot be assured that the transformed ssDNA indeed was the desired sequence, so no statement regarding the efficiency of ssDNA for the transformation and integration into *L. plantarum* can be made. Furthermore the amount of ssDNA used for the integration experiments has been determined by Nanodrop and agarose gel electrophoresis. The ssDNA concentration of both quantification methods differed significantly. This indicates that the used methods are not reliable for quantification of ssDNA. It is possible that an inappropriate amount of ssDNA has been used for the integration experiment, which could explain the failure to generate any transformants. Specific kits design for ssDNA quantification like the Quant-iT™ OliGreen® ssDNA Assay Kit, could overcome this problem. Therefore repeating the transformation and integration experiments with newly generated ssDNA would be recommended.

Only the approach using 5 µg dsDNA resulted in one single chloramphenicol resistant colony. The obtained colony from the dsDNA transformation was investigated to verify the integration into the *cbh* locus of the host *L. plantarum* 3NSH stain. First of all the obtained *L. plantarum* strain was able to grow on MRS-chloramphenicol plates, which indicates the integration of the CAT cassette into the genome. The PCR products of the different primer combinations, which are summarized in figure 47, encourage this statement (figure 48).

The PCR products generated by the primer combination 2-4 showed additional bands of different molecular weight. These bands could be the result of multiple consecutive integration events or unspecific primer interactions with other parts of the isolated genomic DNA. The band at about 1500 bp in figure 48 lane 4 could be an indication for the wild type *cbh* locus. This suggests that the *cbh* gene and its adjacent gene regions occurs multiple times in the genome of the *L. plantarum* 3NSH strain. Another possible explanation for the occurrence of this band could be the presence of wild type bacteria during the DNA isolation.

6.5 Construction of the Flp/*frt* based integration cassette and integration into the genome of *L. plantarum*

The main objective of this thesis was to generate a Flp/*frt* based integration cassette, that consists of the homologous regions of the *cbh* gene, the gene for the cellulase (*celAocES)* under the control of a *efp* promoter (*Lactobacillus buchneri* CD034 *elongation factor P* promoter), a terminator sequence (*L. casei* L-lactat dehydrogenase terminator, T_{ldh}), the chloramphenicol acetyl-transferase (*CAT*) cassette and the *frt* sites for the Flp/*frt* recombination system, and integrate this cassette into the genome of *L. plantarum*. For that reason the Flp/*frt* based integration cassette was generated as summarized in figure 26. The resulting linear Flp/*frt* based integration cassettes (5´cbh_efp_celAocES_fcf3c and 5´cbh_efpshort2_celAocES_fcf3c) were used for transformation into the *L. plantarum strains* CD033 and 3NSH. Like in the previous transformation experiments using the 5´3´cbh_CAT cassette, the integration efficiency of the 5´cbh_efp_celAocES_fcf3c and 5´cbh_efpshort2_celAocES_fcf3c cassettes was very low. No chloramphenicol resistant colony were obtained from the transformation using *L. plantarum* CD033 as host strain while only 2 chloramphenicol resistant colonies were obtained using the *L. plantarum* 3NSH strain. Only one colony obtained from the transformation experiment was able to regrow on MRS-CM plates and showed endoglucanase activity as determined by a Congo red-assay (figure 64). The integration verification of the 5´cbh_efp_celAocES_fcf3c cassette by PCR using the primer combinations summarized in figure 65 suggested that the cassette was

integrated into the *cbh* gene locus (figure 66). Like in the previous integration experiment using the 5´3´cbh_CAT cassette the PCR of some primer combinations (for example figure 66 lane 8) showed additional bands of different molecular weights. Like before these bands could be an indication of multiple consecutive integration events that lead to products of higher molecular weight or unspecific primer interactions. To further investigate the unexpected bands, PCR using the different primer combinations and genomic DNA isolated from the wild type *L. plantarum* 3NSH was performed (figure 66 lanes 3, 6, 9, 12 and 16). Only the expected band of the wild type *cbh* gene (figure 66 lane 9) resulted from these PCR experiments suggesting that the used primer combination only amplified sequences generated during the integration of the 5´cbh_efp_celAocES_fcf3c cassette. However, the template DNA concentration used in these experiments has not been determined. Therefore it is possible that unfavourable DNA/primer rations were used in some of the experiments resulting in no amplification or lower amplification efficiency.

The stability assay and the following PCR verification showed that the integration of the Flp/*frt* based integration cassette into the genome of *L. plantarum* 3NSH is stable for the investigated time period (figure 67). The sequence analysis of the different PCR products generated by the primer combinations summarized in figure 71 confirmed the integration of the 5´cbh_efp_celAocES_fcf3c cassette on the sequence level. The sequencing result showed some single mismatches that could be caused by the error frequency of the used DreamTag polymerase. This polymerase has no proof-reading function which could explain the found mismatches. However the found mismatches are irrelevant for the function of the integrated cellulase because they are locally limited to the 3´end of the *CAT* and the 3`*cbh* sequences. The relevant *frt*-site between the *CAT* sequence and the 3`*cbh* sequences shows no mismatches. Taken together the 5´cbh_efp_celAocES_fcf3c cassette has successfully been integrated into the genome of *L. plantarum* 3NSH.

7 Conclusion and Outlook

One aim of this thesis was to express and purify the gene product of the *ssb* and *recA* genes originating from *L. plantarum* CD033. In this project it was possible to heterologously express the *Lactobacillus* derived recombinant proteins SSBnHis and RecAnHis in *E. coli* BL21DE3 and purify them by using Ni-NTA sepharose column. Additionally the DNA binding capacity of these proteins was assessed using a gel shift assay.

Another aim of this thesis was to generate an Flp/*frt* based integration cassette which can be used for the generation of a *L. plantarum* strain that is able to express the cellulase CelAcES and meet the requirements of food grade microorganisms. During this project it was possible to integrate this Flp/*frt* based integration cassette into the genome of *L. plantarum* 3NSH. To finally generate a food grade microorganism which is necessary in order to use the generated *L. plantarum* in the silage process of modern agriculture, the *CAT* cassette has to be removed from the genome. For this purpose the *frt*-sites were incorporated into the integration cassette. The enzyme Flippase facilitates the site specific recombination of the *frt*-sites with each other, which leads to the removal of one of the *frt*-sites and sequences between them (figure 74).

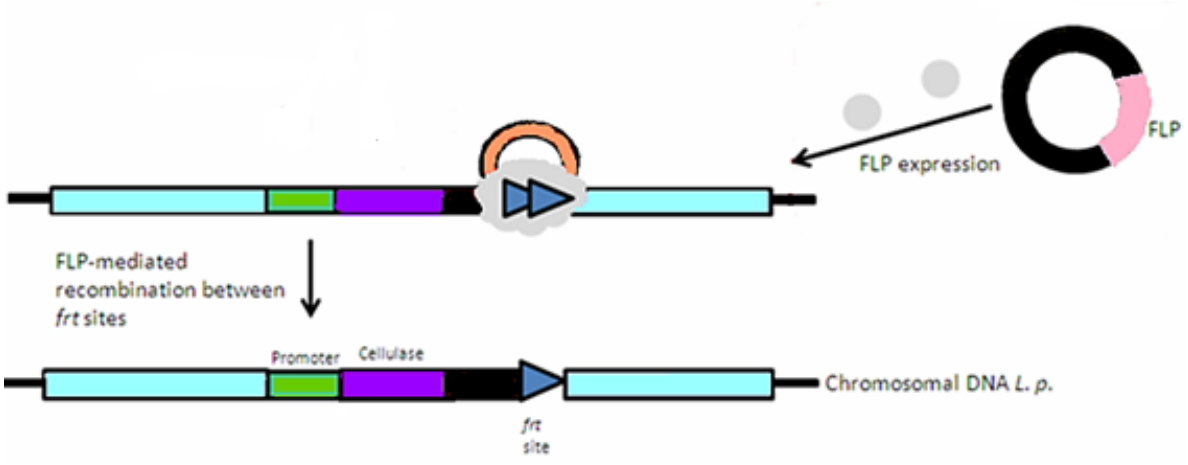

Figure 74: Scheme of the Flp/frt recombination event. *The Flippase (Flp) catalyzes the recombination of the frt-sites, which leads to the excision of the DNA sequence between them (adapted from the presentation "FLP-System" Jan. 2013 © Sandra Nußbaumer).*

Therefore the Flippase is necessary to remove the *CAT* sequence. However transformation of another plasmid that contains the gene encoding the Flippase would again introduce foreign sequences that are not allowed in food grade microorganisms. One possible solution for this problem would be to use a thermosensitive plasmid as described by Yoshida, Terawaki, & Nakaya, 1978 [47] to introduce the Flippase into the *L. plantarum* containing the Flp/*frt* based integration cassette. Once the Flippase removed the chloramphenicol resistance, the *L. plantarum* strain could simply be maintained at a higher temperature for a short period of time. This would lead to the loss of the Flippase containing plasmid and would result in an *L. plantarum* strain with the cellulase CelAcES stably integrated into the genome that also fulfils the requirements of food grade microorganisms.

The comparison of the integration efficiency of linear ssDNA loaded with SSB and RecA, linear ssDNA without protein loading and linear dsDNA, which was also one aim of this thesis, was not possible. Because of several mistakes that happened during the preparation of the linear ssDNA the performed integration experiments were not representative. Further complicating the use of ssDNA is the lack of an appropriate strategy to distinguish ssDNA from dsDNA and to reliable determine the concentration of ssDNA. Therefore it is necessary to establish reliable methods to overcome these problems before the integration of ssDNA compared to dsDNA can be investigated in more detail.

The strategy to heterologously express and purify DNA binding proteins and load them onto linear ssDNA is very time consuming. To avoid this problem, a *L. plantarum* strain that overexpresses SSB and RecA could be generated. This strain could then be used for integration experiments for ssDNA. The loading of the ssDNA would happen intracellular which would significantly reduce the required expenditure to perform ssDNA integration experiments and would most likely result in an optimal protein/DNA ratio. However this strategy has the disadvantage that overexpression of SSB and RecA could have detrimental effects on the growth rate and other important properties of the generated *L. plantarum* strain. Co-transformation of an instable plasmid that encodes the DNA binding proteins together with the linear ssDNA fragments could be a suitable and time efficient strategy to perform ssDNA integration experiments.

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9 Appendix

9.1 Gene- and amino acid sequences

9.1.1 Used homologous regions of the *cbh* gene

5´cbh

TTGGCTCTTAAAGTGTTTGGAATAGATTAAATTATTTTAGAGATGATTTTATGAAAAAAGCTTTATTTTTTCA
GCAACGATATTATACTAAAAAGATGTAAGCTTATTAACTTATCAAATCTAAATATTATTTTCGAGGAGGATT
ACTAGTTATGTGTACTGCCATAACTTATCAATCTTATAATAATTACTTCGGTAGAAATTTCGATTATGAAATT
TCATACAATGAAATGGTTACGATTACGCCTAGAAAATATCCACTAGTATTTCGTAAGGTGGAGAACTTAGA
TCACCATTATGCAATAATTGGAATTACTGCTGATGTAGAAAGCTATCCACTTTACTACGATGCGATGAATGA
AAAAGGCTTGTGTATTGCGGGATTAAATTTTGCAGGTTATGCTGATTATAAAAAATATGATGCTGATAAAG
TTAATATCACACCATTTGAATTAATTCCTTGGTTATTGGGACAATTTTCAAGTGTTAGAGAAGTGAAAAAGA
ACATACAAAAACTAAACTTGGTTAATATTAATTTTAGTGAACAATTACCATTATCACCGCTACATTGGTTGGT
TGCTGATAAACAGGAAT

3´cbh

GGTGTGTTAACAAACAATCCTAATTTTGACTACCAATTATTTAATTTGAACAACTATCGTGCCTTATCAAATA
GCACACCTCAAAATAGTTTTTCGGAAAAAGTGGATTTAGATAGTTATAGTAGAGGAATGGGCGGACTAGG
ATTACCTGGAGACTTGTCCTCAATGTCTAGATTTGTCAGAGCCGCTTTTACTAAATTAAACTCGTTGCCGAT
GCAGACAGAGAGTGGCAGTGTTAGTCAGTTTTTCCATATACTAGGGTCTGTAGAACAACAAAAAGGGCTAT
GTGAAGTTACTGACGGAAAGTACGAATATACAATCTATTCTTCTTGTTGTGATATGGACAAGGGAGTTTATT
ACTATAGAACTTATGACAATAGTCAAATTAACAGTGTCAATTTAAACCATGAGCACTTGGATACGACTGAAT
TAATTTCTTATCCATTACGATCAGAAGCACAATACTATGCAGTTAACTAAAAGCCACTACTGTAATAGTTAA
AATTGTTTAAAAGAGGAAATCAGTTTGTTATCAGTTGATTTCCTCTTTTA

9.1.2 Short linear integration cassette (5´3´cbh_CAT)

TTGGCTCTTAAAGTGTTTGGAATAGATTAAATTATTTTAGAGATGATTTTATGAAAAAAGCTTTATTTTTTCA
GCAACGATATTATACTAAAAAGATGTAAGCTTATTAACTTATCAAATCTAAATATTATTTTCGAGGAGGATT
ACTAGTTATGTGTACTGCCATAACTTATCAATCTTATAATAATTACTTCGGTAGAAATTTCGATTATGAAATT
TCATACAATGAAATGGTTACGATTACGCCTAGAAAATATCCACTAGTATTTCGTAAGGTGGAGAACTTAGA
TCACCATTATGCAATAATTGGAATTACTGCTGATGTAGAAAGCTATCCACTTTACTACGATGCGATGAATGA
AAAAGGCTTGTGTATTGCGGGATTAAATTTTGCAGGTTATGCTGATTATAAAAAATATGATGCTGATAAAG
TTAATATCACACCATTTGAATTAATTCCTTGGTTATTGGGACAATTTTCAAGTGTTAGAGAAGTGAAAAAGA
ACATACAAAAACTAAACTTGGTTAATATTAATTTTAGTGAACAATTACCATTATCACCGCTACATTGGTTGGT
TGCTGATAAACAGGAATGAGCTCTGGTACCGTCGACCTGCAGACTCGAGCCTCGCCGGCAATAGTTACCCT
TATTATCAAGATAAGAAAGAAAAGGATTTTTCGCTACGCTCAAATCCTTTAAAAAAACACAAAAGACCACAT
TTTTTAATGTGGTCTTTATTCTTCAACTAAAGCACCCATTAGTTCAACAAACGAAAATTGGATAAAGTGGGA
TATTTTTAAAATATATATTTATGTTACAGTAATATTGACTTTTAAAAAAGGATTGATTCTAATGAAGAAAGCA
GACAAGTAAGCCTCCTAAATTCACTTTAGATAAAAATTTAGGAGGCATATCAAATGAACTTTAATAAAATTG

ATTTAGACAATTGGAAGAGAAAAGAGATATTTAATCATTATTTGAACCAACAAACGACTTTTAGTATAACCA
CAGAAATTGATATTAGTGTTTTATACCGAAACATAAAACAAGAAGGATATAAATTTTACCCTGCATTTATTTT
CTTAGTGACAAGGGTGATAAACTCAAATACAGCTTTTAGAACTGGTTACAATAGCGACGGAGAGTTAGGTT
ATTGGGATAAGTTAGAGCCACTTTATACAATTTTTGATGGTGTATCTAAAACATTCTCTGGTATTTGGACTCC
TGTAAAGAATGACTTCAAAGAGTTTTATGATTTATACCTTTCTGATGTAGAGAAATATAATGGTTCGGGGAA
ATTGTTTCCCAAAACACCTATACCTGAAAATGCTTTTTCTCTTTCTATTATTCCATGGACTTCATTTACTGGGT
TTAACTTAAATATCAATAATAATAGTAATTACCTTCTACCCATTATTACAGCAGGAAAATTCATTAATAAAGG
TAATTCAATATATTTACCGCTATCTTTACAGGTACATCATTCTGTTTGTGATGGTTATCATGCAGGATTGTTT
ATGAACTCTATTCAGGAATTGTCAGATAGGCCTAATGACTGGCTTTTATAATATGAGATAATGCCGACTGTA
CTTTTTACAGTCGGTTTTCTAATGTCACTAACCTGCCCCGTTAGTTGAAGAAGGTTTTTATATTACAGCTCCA
GATCCATATCCTTCTTTTTCTGAACCGACTTCTCCTTTTTCGCTTCTTTATTCCAATTGCTTTATTGACGTTGAG
CCTCGGAACCCTTAACAATCCCAAAACTTGTCGAATGGTCGGCTTAATAGCTCACGCTATGCCGACATTCTC
GAGCCTGCAGGGTGTGTTAACAAACAATCCTAATTTTGACTACCAATTATTTAATTTGAACAACTATCGTGC
CTTATCAAATAGCACACCTCAAAATAGTTTTTCGGAAAAAGTGGATTTAGATAGTTATAGTAGAGGAATGG
GCGGACTAGGATTACCTGGAGACTTGTCCTCAATGTCTAGATTTGTCAGAGCCGCTTTTACTAAATTAAACT
CGTTGCCGATGCAGACAGAGAGTGGCAGTGTTAGTCAGTTTTTCCATATACTAGGGTCTGTAGAACAACAA
AAAGGGCTATGTGAAGTTACTGACGGAAAGTACGAATATACAATCTATTCTTCTTGTTGTGATATGGACAA
GGGAGTTTATTACTATAGAACTTATGACAATAGTCAAATTAACAGTGTCAATTTAAACCATGAGCACTTGGA
TACGACTGAATTAATTTCTTATCCATTACGATCAGAAGCACAATACTATGCAGTTAACTAAAAGCCACTACT
GTAATAGTTAAAATTGTTTAAAAGAGGAAATCAGTTTGTTATCAGTTGATTTCCTCTTTTA

9.1.3 Flp/*frt* based integration cassette (5´cbh_efp_celAocES_frt_CAT_frt_3´cbh)

TTGGCTCTTAAAGTGTTTGGAATAGATTAAATTATTTTAGAGATGATTTTATGAAAAAAGCTTTATTTTTTCA
GCAACGATATTATACTAAAAAGATGTAAGCTTATTAACTTATCAAATCTAAATATTATTTTCGAGGAGGATT
ACTAGTTATGTGTACTGCCATAACTTATCAATCTTATAATAATTACTTCGGTAGAAATTTCGATTATGAAATT
TCATACAATGAAATGGTTACGATTACGCCTAGAAAATATCCACTAGTATTTCGTAAGGTGGAGAACTTAGA
TCACCATTATGCAATAATTGGAATTACTGCTGATGTAGAAAGCTATCCACTTTACTACGATGCGATGAATGA
AAAAGGCTTGTGTATTGCGGGATTAAATTTTGCAGGTTATGCTGATTATAAAAAATATGATGCTGATAAAG
TTAATATCACACCATTTGAATTAATTCCTTGGTTATTGGGACAATTTTCAAGTGTTAGAGAAGTGAAAAAGA
ACATACAAAAACTAAACTTGGTTAATATTAATTTTAGTGAACAATTACCATTATCACCGCTACATTGGTTGGT
TGCTGATAAACAGGAATGAGCTCTGGTCTTTATTCTTCAACTAAAGCACCCATTAGTTCAACAAACGGATGA
AAAGGACGCGACAAGCGTCAGGTTTCAGTTTATCCAGTTGCTGAAGCTGCAAAGTAAGCACAATAGTTTAA
TTAAAGAGGCTGGGACAAAACGCGTGTTTTGCATCCCGGCTTTTTAATTGCTCAGGATCAGAATGCCATTTT
CATTGGCTATTTTGACCGAAAGTATTATATAATCTATTTATGTTACATTACATTTAGGAGGCATATCAGATCT
ATGAAACGGTCAATCTCTATTTTTATTACGTGTTTATTGATTACGTTATTGACAATGGGCGGCATGATAGCTT
CGCCGGCATCAGCAGCTGGTACTGATGGTACGCCAGTTGAACGTCATGGTCGTTTACGGGTTATGAATGGT
AATATTGTTGGTGAACACGGTAGTCCAGTTCAATTGAAGGGCATGTCATTATTTTGGAGTCAATGGTCAAA
CTACTACAACGGTAACGTTGTTAACAGTTTGGCTGATAACTGGGAATCAACGGTTGTTCGGGCTGCAATGG
GTATTGAAGGTGAAGATGGTTATTTACAAGATGCTGGTGCACAAAAAGCCAAGGCGAAAACTATTGCGGA
TGCCGCGATTGCTAAGGGTATTTACGTTATTTTGGATTGGCATGATCACAATGCACATCAACACTTAGATTT
GGCCAAGAGTTATTTTCGTGAAGTTGCTCAAGCATACAAGAACACTCCAAACGTTATTTTTGAAGTTTTTAA
TGAACCATTGAATACAAATACCTGGCCAGCCGTTAAAAGTTATGCCGAAGCGGTTATTTCAGAAATTCGGG
GTCAAGGTGCTAACAACTTGGTTATTGTTGGTAGTCCAAATTGGTCACAAGATGTTGATATTGCTGCTGATA

ATCCATTGAGTGATCAAAACGTTGCATACACTTTGCATTTTTATGCAAATACGCACAAGGCCTCATTGCGTG
ATAAGGCACAAAAGGCCATTAACAAGAAGTTGGCGTTGTTTGTTACAGAATGGGGTACCTGTAGTGCCGA
TGGTAATGGTCAATTAAATTTGGGTGAATCACAAACTTGGTTGGATTTCTTAGATAGTCATAACATTTCATG
GGCTAACTGGAGTTTGGGTGATAAGGCTGAAGCATGTTCAGCGTTACGGCCAAATGCTAATCAAATGGGT
AACTGGAACGATAACGATTTGACGGAAAGTGGCAAGTGGGTTAAGGCGAAAATTGCTGAATAACTGCAGC
AGGTCGACAAAACCGCTGTCCGAGACCGCGCGTCACAACGCGGGCAATCTCAGGCAGCGGCTTTTTTAATC
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GATATTTTTAAAATATATATTTATGTTACAGTAATATTGACTTTTAAAAAAGGATTGATTCTAATGAAGAAA
GCAGACAAGTAAGCCTCCTAAATTCACTTTAGATAAAAATTTAGGAGGCATATCAAATGAACTTTAATAAA
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ACCACAGAAATTGATATTAGTGTTTTATACCGAAACATAAAACAAGAAGGATATAAATTTTACCCTGCATTT
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AGGTTATTGGGATAAGTTAGAGCCACTTTATACAATTTTTGATGGTGTATCTAAAACATTCTCTGGTATTTG
GACTCCTGTAAAGAATGACTTCAAAGAGTTTTATGATTTATACCTTTCTGATGTAGAGAAATATAATGGTTC
GGGGAAATTGTTTCCCAAAACACCTATACCTGAAAATGCTTTTTCTCTTTCTATTATTCCATGGACTTCATTTA
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TAAAGGTAATTCAATATATTTACCGCTATCTTTACAGGTACATCATTCTGTTTGTGATGGTTATCATGCAGGA
TTGTTTATGAACTCTATTCAGGAATTGTCAGATAGGCCTAATGACTGGCTTTTATAATATGAGATAATGCCG
ACTGTACTTTTTACAGTCGGTTTTCTAATGTCACTAACCTGCCCCGTTAGTTGAAGAAGGTTTTTATATTACA
GCTCCAGATCCATATCCTTCTTTTTCTGAACCGACTTCTCCTTTTTCGCTTCTTTATTCCAATTGCTTTATTGAC
GTTGAGCCTCGGAACCCTTAACAATCCCAAAACTTGTCGAATGGTCGGCTTAATAGCTCACGCTATGCCGAC
ATTGGATCCGCATGCGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGTGTGTTAACAAACAATCCTAA
TTTTGACTACCAATTATTTAATTTGAACAACTATCGTGCCTTATCAAATAGCACACCTCAAAATAGTTTTTCG
GAAAAAGTGGATTTAGATAGTTATAGTAGAGGAATGGGCGGACTAGGATTACCTGGAGACTTGTCCTCAA
TGTCTAGATTTGTCAGAGCCGCTTTTACTAAATTAAACTCGTTGCCGATGCAGACAGAGAGTGGCAGTGTT
AGTCAGTTTTTCCATATACTAGGGTCTGTAGAACAACAAAAAGGGCTATGTGAAGTTACTGACGGAAAGTA
CGAATATACAATCTATTCTTCTTGTTGTGATATGGACAAGGGAGTTTATTACTATAGAACTTATGACAATAG
TCAAATTAACAGTGTCAATTTAAACCATGAGCACTTGGATACGACTGAATTAATTTCTTATCCATTACGATCA
GAAGCACAATACTATGCAGTTAACTAAAAGCCACTACTGTAATAGTTAAAATTGTTTAAAAGAGGAAATCA
GTTTGTTATCAGTTGATTTCCTCTTTTA

9.1.4 SSB

ssbnhis

ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGATTAACCG
TACAATTCTTGTTGGCCGACTGACAAGAGATCCGGAATTACGTTACACGAATGGTGGTGCCGCCGTCGCGA
CGTTCACTATTGCCGTAAACCGTCAATTCACGAATCAAAATGGGGAACGTGAAGCAGATTTCATTAGTTGC
GTCATTTGGCGTAAAGCTGCTGAAAATTTTGCGAACTTTACTCATAAAGGTTCACTTGTTGGTATCGATGGC
CGGATTCAAACCCGGAACTACGAAAACCAACAAGGGGTACGTGTTTACGTTACAGAAGTTGTCGTTGAAA
ACTTCTCATTATTAGAATCACGGGCAGAGTCTGAACGTCATCAGTCTGCCAATGGTGGTAGTGGTAATAAC
AATTACAACAATGGTAATTCGAATTACAACAACAATAATAATGGGTATAGTAATCAAGGCCAAAATGCGGC
TCCTCAACAATCATCAGCAAATAACAATAACCCATTTGGTAATGGTAATACTGGTAATGCTAGCAGTGCGG
CACCATCAAGCAGTGCTAACAACAATAATCAGGCTGACCCATTCGCTAATAATGGCGATCAAATTGATATCT
CGGATGATGATTTACCATTCTAG

SSBnHis

MGSSHHHHHHSSGLVPRGSHMINRTILVGRLTRDPELRYTNGGAAVATFTIAVNRQFTNQNGEREADFISCVI
WRKAAENFANFTHKGSLVGIDGRIQTRNYENQQGVRVYVTEVVVENFSLLESRAESERHQSANGGSGNNNYN
NGNSNYNNNNNGYSNQGQNAAPQQSSANNNNPFGNGNTGNASSAAPSSSANNNNQADPFANNGDQIDIS
DDDLPF

9.1.5 RecA

recAnhis

ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGGCTGATG
CACGGAAAGCAGCACTAGATACTGCCCTGAAAAAGATCGAAAAGAATTTCGGTAAAGGGGCGATTATGCG
GATGGGTGACGCTGCCCAGACGACTATTTCAACGATTTCAAGTGGATCGTTGGCCTTAGATGACGCGTTGG
GTGTCGGCGGCTACCCACGTGGTCGGATCGTGGAAATCTACGGTCCTGAAAGTTCAGGTAAAACGACCGT
GGCACTACATGCGGTTGCTGAAGTTCAAAAGCAGGGTGGTACGGCGGCCTATATCGATGCTGAAAACGCA
CTAGACCCCGTTTATGCGGAACACCTAGGGGTCAACATTGATGACCTGTTACTTTCGCAACCAGATACTGGT
GAACAAGGGCTTGAAATTGCAGATGCCTTAGTTTCCAGTGGTGCGGTCGATATTTTAGTTGTTGACTCGGT
GGCGGCCTTAGTGCCACGTGCCGAAATTGAAGGTGAAATGGGTGACGCACACGTTGGGTTACAAGCGCG
GCTGATGTCACAAGCGCTCCGGAAGTTATCAGGGACATTGAACAAAACCAAGACAATCGCGTTATTTATCA
ATCAAATTCGTGAAAAAGTTGGTGTGATGTTTGGTAATCCTGAAACGACTCCTGGTGGTCGGGCCTTGAAA
TTCTACGCCACGATTCGTTTGGAAGTACGGCGAGCAGAACAGATCAAGGAAGGAACCAATATTATTGGTA
ACCGTGTCCGGATCAAAGTTGTTAAGAACAAGGTTGCACCGCCGTTTAAGCGTGCCGAAGTGGATATCATG
TATGGTCAAGGTATCTCACAAACTGGTGAAATTGTTGATATGGCTGCTGAAAAGGATATTGTTAAGAAGAG
TGGTTCTTGGTATTCATATGGTGACGATCGCATTGGTCAAGGCCGTGAAAATGCCAAGAAGTATTTGGACG
AGCATCCTGATGTCATGACGGAGATTCGCCAAAAGGTTCGTGATGCATACGGTATGGATGCAACTGGTGA
AGAAACGTCTGAAACTGATGATCAGGCCAAAGAAGCTAAGGATAAGGGAACTGCTAAGAATGGCAGTAA
GGGTCAAAGTAAGTCCACTAAAGCGACACCAGCGGAAACTGCTCTCGATCTCGGCGACCAACCAACCGAA
AAAAAGCTTGGCTGTTTTGGCGGATGA

RecAnHis

MGSSHHHHHHSSGLVPRGSHMADARKAALDTALKKIEKNFGKGAIMRMGDAAQTTISTISSGSLALDDALGV
GGYPRGRIVEIYGPESSGKTTVALHAVAEVQKQGGTAAYIDAENALDPVYAEHLGVNIDDLLLSQPDTGEQGLEI
ADALVSSGAVDILVVDSVAALVPRAEIEGEMGDAHVGLQARLMSQALRKLSGTLNKTKTIALFINQIREKVGVM
FGNPETTPGGRALKFYATIRLEVRRAEQIKEGTNIIGNRVRIKVVKNKVAPPFKRAEVDIMYGQGISQTGEIVDM
AAEKDIVKKSGSWYSYGDDRIGQGRENAKKYLDEHPDVMTEIRQKVRDAYGMDATGEETSETDDQAKEAKDK
GTAKNGSKGQSKSTKATPAETALDLGDQPTEKKLGCFGG

9.1.6 *efp* promoter versions

Table 25: Sequences of the efp promoter versions

promoter	5´-3´Sequence
P_{efp}	TGGTCTTTATTCTTCAACTAAAGCACCCATTAGTTCAACAAACGGATG AAAAGGACGCGACAAGCGTCAGGTTTCAGTTTATCCAGTTGCTGAAG CTGCAAAGTAAGCACAATAGTTTAATTAAAGAGGCTGGGACAAAACG CGTGTTTTGCATCCCGGCTTTTTAATTGCTCAGGATCAGAATGCCATTT TCATTGGCTATTTTGACCGAAAGTATTATATAATCTATTTATGTTACAT TACATTTAGGAGGCATATCAGATCT
short version 1 of the promoter P_{efp}	GAAAAGGACGCGACAAGCGTCAGGTTTCAGTTTATCCAGTTGCTGAA GCTGCAAAGTAAGCACAATAGTTTAATTAAAGAGGCTGGGACAAAAC GCGTGTTTTGCATCCCGGCTTTTTAATTGCTCAGGATCAGAATGCCAT TTTCATTGGCTATTTTGACCGAAAGTATTATATAATCTATTTATGTTAC ATTACATTTAGGAGGCATATCAGATCT
short version 2 of the promoter P_{efp}	GCACAATAGTTTAATTAAAGAGGCTGGGACAAAACGCGTGTTTTGCA TCCCGGCTTTTTAATTGCTCAGGATCAGAATGCCATTTTCATTGGCTAT TTTGACCGAAAGTATTATATAATCTATTTATGTTACATTACATTTAGGA GGCATATCAGATCT

9.2 Strain collection

Table 26: Assignment of the used E. coli strains and L. plantarum strain to the IMBT strain collection number

IMBT strain collection	Strain	Plasmid
#7418	*E. coli* XL1-Blue	pJET_5´cbh_efp_celAocES_fcf3c
#7419	*E. coli* XL1-Blue	pJET_5´cbh_efpshort2_celAocES_fcf3c
#7420	*E. coli* XL1-Blue	pMS470_5´3´cbh_CAT
#7417	*E. coli* XL1-Blue	pMS470nHis_ssb
#7422	*L. plantarum* 3NSH	5´cbh_efp_celAocES_fcf3c (Integrant)