University of Natural Resources and Applied Life Sciences, Vienna

Department of Biotechnology

Institute of Applied Microbiology (IAM)

Head: o. Univ. Prof. Dr. Hermann Katinger

Supervisor: ao. Univ. Prof. Dr. Reingard Grabherr

Assistant Supervisor: DI Dr. Stefan Heinl

VECTOR-FREE CLONING AND HETEROLOGOUS EXPRESSION OF AN ENDO-1,4-B-GLUCANASE FROM *BACILLUS SUBTILIS* IN *LACTOBACILLUS PLANTARUM*

Master Thesis

Submitted by Katharina Spath

Vienna, December 2008

Acknowledgement

First of all my thanks are entitled to A.o.Univ.Prof.Dipl.-Ing. Dr. Reingard Grabherr for giving me the opportunity to perform my master thesis in the BACULO group. Thank you for always having an open door and mind for our problems and for creating this constructive atmosphere.

I especially want to thank my supervisor Stefan Heinl for his assistance and for sharing his knowledge and experience with me.

I would like to thank the members of the BACULO group, namely Stefan Gross, Didi Palmberger, Jens Pontiller, Jürgen Mairhofer, Florian Krammer, Esther Egger, Martina Baumann and Christopher Tauer for their support and friendly atmosphere in our lab. I enjoyed working with you!

Further I want to thank my student colleagues, especially Silfa, Kathi, Alba and Andi for always being there for me and for their friendship.

My sincere gratitude goes out to my family for their incessant support throughout my studies.

Abstract

Lactic acid bacteria play an important role in the preservation of ensiled forages from microbial and enzymatic deterioration. The efficiency of preservation depends on the rapidity of acidification by lactic fermentation. The rate of acidification is constrained by the concentration of water-soluble carbohydrates in the row plant material as they are the growth limiting factor for lactic acid bacteria. A possibility to overcome this growth limitation is, to provide lactic acid bacteria with enzymes that are able to hydrolyse complex carbohydrates, as for example cellulases, amylases or xylanases.

In this master thesis a commercial *Lactobacillus plantarum* strain was genetically modified by integration of an endo-1,4-β-glucanase gene from *Bacillus subtilis* into the host chromosome by a vector free cloning technique. Therefore, a transfer vector permitting vector free cloning was constructed. For the transformation an optimized electrotransformation protocol for the *Lactrobacillus plantarum* strain was established. Integration of the transfer vector into the chromosome and elimination of unwanted sequences was performed by two step homologous recombination. The heterologous gene was inserted into the *cbh* gene, which encodes the non-essential conjugated bile hydrolase. The obtained recombinant strain *Lactobacillus plantarum* E-1,4-BG, contains no more unwanted vector sequences and the activity of the endo-1,4-β-glucanase was demonstrated by Congo red assay.

Zusammenfassung

Milchsäurebakterien spielen bei der Konservierung von silierten Futtermitteln eine wichtige Rolle und schützen diese vor mikrobiellem und enymatischem Abbau. Die Effizienz der Konservierung hängt von der Geschwindigkeit der Ansäuerung durch die Produktion von Milchsäure ab. Die Geschwindigkeit der Ansäuerung ist wiederum abhängig von der Konzentration von wasserlöslichen Kohlenhydraten im Pflanzenmaterial, welche der limitierende Faktor für das Wachstum von Milchsäurebakterien ist. Eine Möglichkeit diese Wachstumslimitierung zu umgehen ist, die Bakterien mit Enzymen auszustatten, die fähig sind komplexe Kohlenhydrate zu hydrolysieren, wie zum Beispiel Cellulasen, Amylasen oder Xylasen.

In dieser Diplomarbeit wurde ein kommerzieller *Lactobacillus plantarum* Stamm durch Integration einer Endo-1,4-β-glucanase aus *Bacillus subtilis* in das Wirtschromosom, genetisch modifiziert. Zu diesem Zweck wurde ein Transfervektor der eine vektorfreie Klonierung zulässt, konstruiert. Um die Transformationseffizienz zu erhöhen war es notwendig ein optimiertes Elektrotransformationsprotokoll zu etablieren. Mittels zweistufiger homologer Rekombination wurde der Transfervektor in das Chromosom von *Lactobacillus plantarum* integriert und unerwünschte Sequenzen wieder entfernt. Das heterologe Gen wurde dabei in das *cbh* Gen, welches für eine nicht essentielle dekonjugierende Gallensäurehydrolase kodiert, integriert. Der rekombinante Stamm *Lactobacillus plantarum* E-1,4-BG enthält nunmehr keinerlei unerwünschte Sequenzen und die Aktivität der Endo-1,4-β-glucanase wurde durch einen Congorot Test nachgewiesen.

1 Introduction

1.1 Lactic acid bacteria

The group of lactic acid bacteria (LAB) subsumes Gram-positiv, aero-tolerant, anaerobic, acid-tolerant and non-spore-forming bacteria which have a low G+C content and an ability to produce lactic acid (lactate) by homo- or hetero-fermentative carbohydrate metabolism. Homo-fermentative LAB produce up to 90% pure lactate. They degrade glucose throught the Embden-Meyerhoff-Parnas (EMP) pathway leading to pyruvate, which is subsequently converted to approximately equimolar amounts of D- and L-lactate by stereospecific NAD-dependent lactate dehydrogenases (LDHs). Only a small amount of pyruvate gets decarboxylated and transformed to other metabolites, including formate, acetate, ethanol, acetoin and 2,3-butanediol (Kleerebezem et al. 2003). whose distribution depends on strain and culture parameters. For instance, production of acetate from pyruvate via acetylphosphate has been reported for *L. plantarum* grown on glucose under aerobic conditions (Götz et al. 1980b, Götz et al. 1980a). Heterofermentative lactic acid bacteria (LAB) such as *Leuconostoc*, *Oenococcus*, and *Lactobacillus* strains ferment pentoses by the phosphoketolase pathway.

LAB include several genera of bacteria, including *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus*, *Pediococcus*, *Carnobacterium*, *Enterococcus, Oenococcus* and *Bifidobacterium*. They are morphologically not similar but are alike physiologically. Most LAB are rod shaped but they can also be coccoid. A further characteristic of LAB is their high demand for nutrients. As a consequence of their specialization in the growth in milk and other nutrients riche habitats, they lost the ability to synthesize a lot of metabolites. Beside vitamins (lactoflavine, thiamine, pantothenic acid, nicotinic acid, folic acid, biotin) they also need amino acids, purins and pyrimidines. Because their metabolism is crippled they are cultivated predominantly on complex culture medium containing a high amount of yeast extract, tomato juice, whey or blood.

With their high nutrient requirement and specialized carbohydrate utilization, LAB are found in the gastrointestinal tract and mucosa of mammals, in living and decomposing plants and in milk.

LAB play an important role in industrial feed and food fermentations, where they are used in a variety of food- and feed-stuff such as meat products, milk products, fermented vegetables or other fermented plant materials. Their main function is the preservation by acidification or for improving flavour and texture. To prevent an uncontrolled fermentation process and to get reproducible acidification, so-called starter cultures are added to the fermenting material. Starter cultures are well characterized cultures ensure rapid acidification, bacteriophage resistance. Because of the economic importance of lactic acid bacteria, there is a growing interest in the development of new strains with improved properties.

1.1.1 Lactobacillus plantarum

Lactobacillus plantarum (L. plantarum) is a facultative hetero-fermentative bacterium. Its natural habitats are anaerobic or micro-aerobic and are rich in nutrients and carbohydrates. The complete genome sequence of *L. plantarum* strain WCFS1 was determined (Kleerebezem et al. 2003). The chromosome contains approximately 3.000 protein-encoding genes of which 70% shows putative biological functions. *L. plantarum* WCFS1 also contains two small cryptic plasmids (2,365 and 1,917 bp) and a larger plasmid (36,069 bp) encoding genes involved in conjugal plasmid transfer. To potentially highly expressed (PHX) genes from *L. plantarum* contain genes of the complete EMP pathway, a number of enzymes involved in the degradation of pentoses and hexoses and a number of phosphtransferase systems (PTS). The route of N-acetylglucosamine catabolism in WCFS1 is also entirely PHX.

L. plantarum occurs in many different environmental niches and is a flexible and versatile species because of its ability to grow on a wide variety of sugar sources. This phenotypic trait is reflected by the high number of sugar transporters. *L. plantarum* WCFS1 genome encodes 30 transporter systems which are involved in the transport of carbon sources. For some carbon-uptake systems, no substrate specificity could be predicted and some sugar transporter systems are known to import more than one substrate.

L. plantarum is grouped among the facultative hetero-fermentative LAB. It can degrade sugars via the EMP pathway or via the phosphoketolase pathway, leading to homolactic and heterolactic fermentation profiles (Kandler 1983). During growth on glucose, *L. plantarum* displays an almost homolactic fermentation pattern, in which glucose is degraded via the EMP. The genes for the EMP pathway were found to be organized in two operons. The regulation of expression of these enzymes depends on the level and type of sugar source available in the environment. An intact citrate acid cycle is not encoded on the *L. plantarum* WCFS1 chromosome.

L. plantarum WCFS1 has 19 genes encoding intracellular peptidases of different specificity for degrading peptides. In contrast to *Lactococcus lactis*, which is the most common model strain, *L. plantarum* WCFS1 has three peptidases capable of cleaving N-terminal proline residues whereas *Lactococcus lactis* has none.

Transport proteins are the largest class of proteins in *L. plantarum* WCFS1. 57 ATP-binding cassette (ABC) transporters were identified, of which 27 were importers. These importers transport amino acids and peptides. The other 30 ABC transporters were identified as exporters, but the substrate specificity of most of the exporters is unknown.

The chromosome of *L. plantarum* WCFS1 encodes several stress-related proteins, including some proteases involved in the stress response which degrade aberrant and non-functional proteins. Furthermore the chromosome encodes three small heat shock proteins of the HSP20 family (Van Montfort et al. 2001) and three highly homologous cold shock proteins (Derzelle et al. 2000).

L. plantarum WCFS1 is able to consume oxygen under certain circumstance, including glucose limitation. The absence of the superoxide dismutase in *L. plantarum* WCFS1 has found to be compensated by the capacity of this bacterium to accumulate very high concentrations of intercellular Mn^{2+} ions (20-30 mM), which can act as a scavenger for oxygen radicals (Archibald & Fridovich 1981). There are also genes encoding various oxidative stress-related proteins such as thiol peroxidase, halo peroxidase, glutathione peroxidase, catalase, four glutathione reductases, five NADH-oxidases and two NADH peroxidases, allowing the bacterium to grow under aerobic conditions.

L. plantarum is frequently used as inoculum in meat products, fermentation of vegetable and in grass silage. It converts sugar quantitatively into lactic acid, allowing preservation of these products as an effect of decreasing the pH.
L. plantarum is also found in the human gastrointestinal tract and oral cavity and some strains show probiotic effects on human and animal health (Ahrné et al. 1998). Because of their ability to persist in the human gastrointestinal tract, *L. plantarum* can be used as a delivery vehicle for therapeutic compounds, including vaccines (Seegers 2002).

1.2 Genetically modified lactic acid bacteria

In recent years LAB have been genetically manipulated to enhance their metabolic potential. They were genetically engineered for the production of metabolites, such as L-alanine (Hols et al. 1999), L-ribulose (Helanto et al. 2007) and sorbitol (Ladero et al. 2007), or to change concentration of metabolic intermediates or end-products (Kleerebezem & Stams 2000). Two main advantages of Gram-positive bacteria are a very efficient secretory pathway and a significantly higher solubility of recombinant proteins in contrast to *Escherichia coli* expression systems (Straume et al. 2006). Therefore, LAB have potential for the production of antigens, antibodies, growth factors and anti-microbial peptides called bacteriocins.

In view of the industrial and medical importance of LAB, improvement of strains properties is of major interest. Transformation of industrial strains is more difficult than of laboratory strains because genetically modified organisms (GMO) used in food- and feed-stuff must fulfill a number of safety criteria and must reach the generally regarded as safe (GRAS) status. The definition of GRAS is that a food grade GMO can only contain DNA from the same species or from another GRAS micro organism. In both cases, antibiotic resistance genes as selection markers are not allowed and it is necessary to eliminate all foreign DNA.

Food grade genetic modifications include for example gene deletion (Johansen et al. 1995), gene replacement with a similar gene from another strain (Johansen et al. 1995), gene replacement with the same gene that has been modified in vitro (Sørensen et al. 2000), increasing the number of copies of a gene (Dickely et al. 1995) and introducing a new gene into the strain (Joutsjoki et al. 2002). Most of these modifications require at least temporarily the introduction of plasmid DNA.

1.3 Gene transfer into lactic acid bacteria

Introduction of DNA into LAB strains by protoplast transformation (Simon et al. 1986), protoplast transfection (Boizet et al. 1988) and conjugal transfer (Vescovo 1983), have been described in several reports. These methods for gene transfer for the strains of Lactobacillus have shown low efficiency and lack of reproducibility (de Vos & Vaughan 1994).

Electroporation has become the most commonly used method for introducing DNA in LAB cells. Transformation by electroporation involves a short, high voltage electrical pulse to a suspension of cells and DNA. The electrical pulse results in a rearrangement of the components of the cell wall and membrane to generate transient pores through which the DNA can pass into the cell.

Several protocols for the electrotransformation of different *Lactobacillus* strains have been published and it turned out that only selected strains could be transformed with high efficiency. But most LAB strains take up exogenous DNA at very low frequency. Because of the strain variations, it is necessary to optimize the electroporation conditions for every single LAB strain to obtain efficient genetic transfer.

The efficiency of electrotransformation is affected by a number of parameters including the growth phase at which cells are harvested, cell density, composition of the wash and electroporation solutions, the inclusion of cell wall weakening agents in the growth medium and electric conditions such as electrical pulse strength and length.

The efficiency of electrotransformation is correlated to the level of electropermeabilization (Sixou et al. 1991). In order that an adequate amount of DNA can enter the cell, the physical barrier of the cell wall has to be weakened. This can be achieved by pre-treatment with chemicals that increase cell wall permeability. The inclusion of cell wall weakening agents into growth medium has been shown to improve the transformation rate. Pre-treatment with lysozyme, glycine or penicillin has been

proposed by Wei and Rush (Wei & Rush 1995) Aukrust and Blom (Aukrust & Blom 1992) reported that the highest transformation efficiency was obtained at glycine concentration of 1% whereas Wells *et al.* (Wells et al. 1993) found that 2.5% glycine leads to improved transformation efficiency. Thompson and Collins (Thompson & Collins 1996) reported a high transformation efficiency at the glycine concentration of 8%, whereas Serror *et al.* (Serror et al. 2002) found 0.1% was enough to improve the transformation efficiency.

There are also reports of pre-treatment of LAB with lithium acetate (LiAc) and dithiothreitol (DDT). Treatment of *Lactococcus lactis* cells with LiAc and DDT prior to electroporation resulted in an improvement in transformation efficiency (Papagianni et al. 2007).

This data suggest that the effect of cell wall weakening agents depends on concentration and is highly strain specific.

Another factor in transformation by electroporation is the growth phase at which cells are harvested. Serror *et al.* (Serror et al. 2002) reported that the transformation efficiency was higher when the cells were harvested at the beginning of the stationary phase (OD_{600} 1.7), whereas cells harvested in the exponential phase (OD_{600} 0.2-1.0) or in the stationary phase (OD_{600} >2.0) yielded lower transformation efficiencies. Aukrust and Blom (Aukrust & Blom 1992) has been observed that it is more effective when exponential cultures (OD_{600} 0.6) are used.

The composition of the wash and electroporation solutions is also a factor of influence. Two electroporation solutions 30% PEG_{1500} and 952 mM sucrose with 3.5 mM $MgCl_2$ were used by Aukrust and Blom (Aukrust & Blom 1992). Electroporation in 30% PEG_{1500} was shown to result in higher transformation rates than electroporation in 952 mM sucrose with 3.5 mM $MgCl_2$. There is also a report, that addition of sucrose, an osmotic stabilizer, to the wash solution improved the transformation efficiency, whereas no increase in transformation efficiency was observed with $MgCl_2$ (Serror et al. 2002).

Electrical parameters are the most important factors in transformation by electroporation. To have the highest transformation efficiency and minimum destruction of cells, an optimization of the electrical conditions is necessary. In *Lactobacilli* high transformation efficiencies were obtained at relatively high electric field strength (8-12.5 kV cm^{-1}) (Serror et al. 2002); (Kim et al. 2005). In *Lactococci* better transformation efficiencies were obtained at lower electrical field strength of 6 kV cm^{-1} (Holo & Nes 1989) indicating that an optimization of conditions for electrotransformation is necessary for each species of LAB.

1.4 Homologous recombination

1.4.1 Homologous recombination by single cross-over

Homologous recombination is a simple method to reach chromosomal integration. It is obtained when a non-replicating plasmid, containing regions of homology with the chromosome is introduced into the host as illustrated in figure 1.1 (de Vos 1999). This gene integration is also known as single cross-over or Campbell like integration. The efficiency depends on the size and sequence of the region of homology and from the recombination efficiency of the host. The recombination event is reversible and a single cross-over shows instability. This problem can be solved by applying continuous selective pressure.

1.4.2 Homologous recombination by double cross-over

If two regions of homology on the plasmid are closely linked then, depending on the sequence between the homologous regions chromosomal deletion or the insertion of a gene of interest can occur (Figure 1.2).

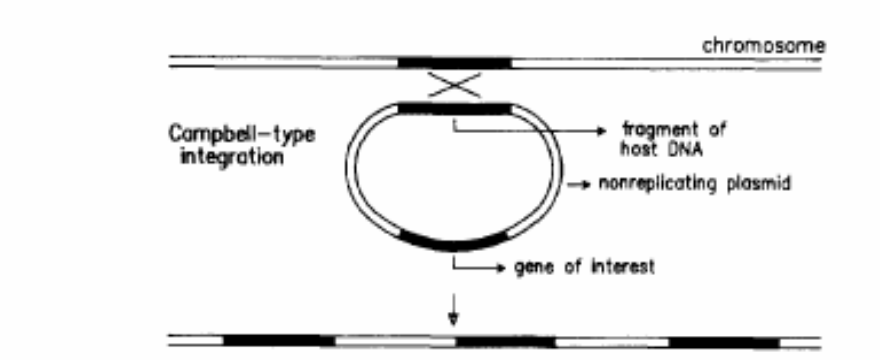


Figure 1.1: Figure illustrates the Campbell like integration. A non-replicating plasmid with a region of homology to the host chromosome integrates by a single cross-over. Thereby the gene of interest is integrated together with the whole plasmid DNA. (Figure from Venema 1993)

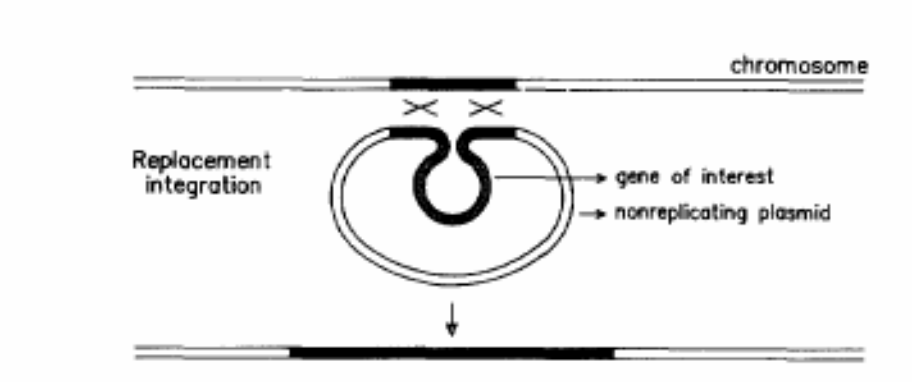


Figure 1.2: Replacement recombination. On the non-replicating plasmid the gene of interest is inserted into the region of homology. A double cross-over leads to an interrupted DNA sequence on the chromosome. (Figure from Venema 1993)

A problem is that gene replacements are difficult to obtain because the gene to be replaced could be essential for the host or there are polar effects in the chromosome due to the insertion of the gene of interest.

1.4.3 Two step homologous recombination

In order to generate food grade GMO, vector free cloning is often preferred. By this cloning method the plasmid DNA, which often contains an antibiotic resistance gene, has to be removed from the chromosome after the first homologous recombination. This could be achieved by a second step of homologous recombination as shown in figure 1.3 A. But it can also happen that a preferred recombination occurs at the homology region where the first step of homologous recombination occurred, resulting in the deletion of the inserted DNA, and subsequent in the wild-type genotype as shown in figure 1.3 B.

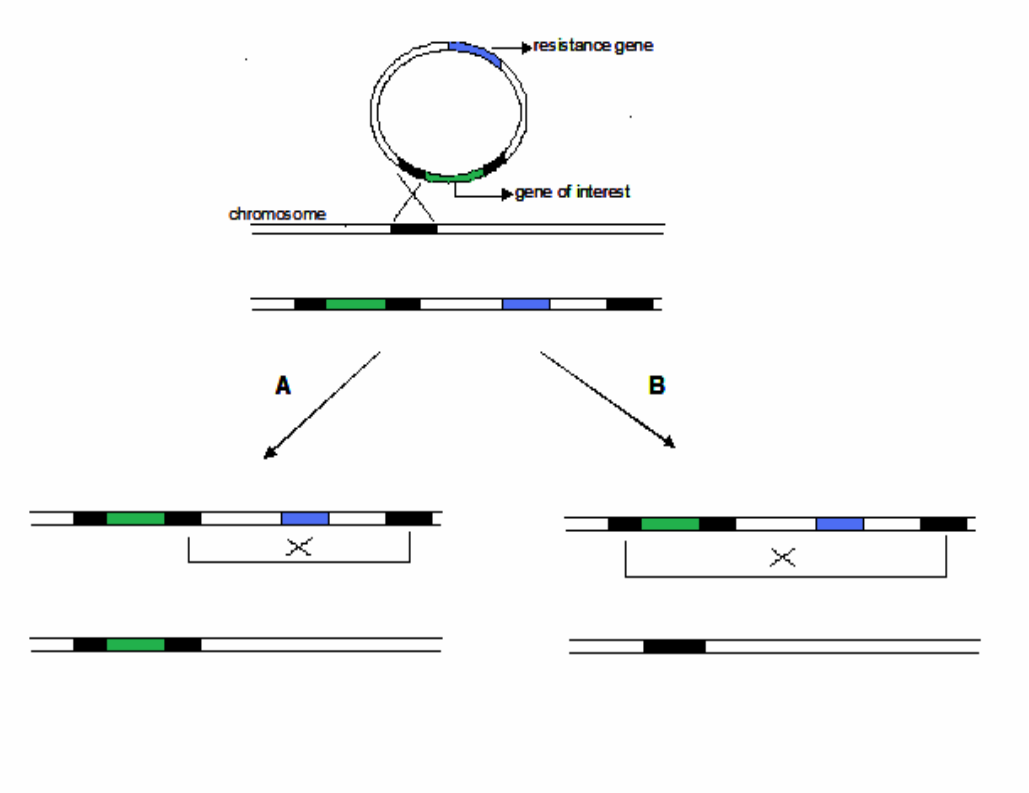


Figure 1.3: Two step homologous recombination: **A)** After the second cross-over the plasmid DNA is removed and only the gene of interest remains on the chromosome. **B)** By the second step of homologous recombination the plasmid DNA together with the gene of interest is removed resulting in the wild-type genotype.

1.5 Heterologous gene expression in LAB

High level production of recombinant proteins in LAB has been obtained using constitutive or inducible promoters (Kuipers et al. 1997). Continuous high level production of a protein could lead to intracellular accumulation, aggregation, or degradation of this protein in the cytoplasm (Makrides 1996). To prevent negative effects caused by high production, inducible promoters have been developed. Gene expression can be controlled by an inducer, a repressor or by environmental factors such as temperature (Nauta et al. 1997), pH or ion concentration. In *Lactococcus lactis*, promoters were identified which are specifically induced by Cl^- (Sanders et al. 1998) or by Zn^{2+} (Llull & Poquet 2004) and the P170 promoter which is a strong promoter, only active at a low pH (Madsen et al. 1999).

A good characterized controllable expression system is based on the lactose inducible transcription of the lac operon (de Vos & Vaughan 1994).
Another inducible expression system has been developed in *Lactococcus lactis*, based on the lactococcal bacteriophage system. Components of this system include a phage origin of replication and phage expression signals, which were induced to high efficiency upon phage infection of the host (O'Sullivan et al. 1996).

The best characterized gene expression system is probably the nisin-controlled expression system (NICE) derived from *Lactococcus lactis*. Many LAB produce antimicrobial peptides called bacteriocins. Their production is regulated via quorum-sensing mechanisms based on secreted peptide pheromones (Eijsink et al. 2002). The pheromone activates a two-component regulatory system consisting of a histidine kinase (NisK) that sense the pheromone, and an intracellular response regulator (NisR) that induces the promoters *nisA* and *nisF* of the operons involved in bacteriocin production. In strains producing class I bacteriocins (lantibiotics such as nisin), the bacteriocin itself acts as pheromone. The transferable NICE system consists of an expression vector carrying the gene of interest under control of the *nisA* promoter and a regulatory plasmid carrying the *nisRK* regulatory genes (Pavan et al. 2000). The

expression of the gene of interest can be induced by the addition of sub-inhibitory amounts of nisin to the culture medium (de Ruyter et al. 1996).

For *Lactobacillus sakei*, an inducible expression system based on promoters and regulatory genes involved in the production of the class II bacteriocins sakacin A or sakacin P has been developed. Strains producing class II bacteriocins produce and secrete a separate, non-modified peptide pheromone. The regulated bacteriocin promoters have been used to construct one-plasmid inducible expression systems employing the so-called pSIP expression vectors (Axelsson et al. 2003); (Sørvig et al. 2005). In these vectors expression of the gene of interest is placed under control of a strong inducible bacteriocin promoter and gene expression is induced by an externally added peptide pheromone called inducing factor (IF).

Although LAB are food-grade organisms, this status can be compromised by the expression systems based on high copy number plasmids, antibiotic resistance genes as selection markers and the use of foreign DNA. Food grade cloning systems have been developed to efficiently produce proteins directly in food or in large scale fermentations. In order to avoid the presence of resistance gene after genetic manipulation, integration of foreign genes into the host chromosome represents a good option for stable maintaining cloned genes without the need for selection markers (Gosalbes et al. 2000).

Chromosomal integration is based on non- or conditionally-replicating plasmids. Many non-replicating plasmids have replicons of Escherichia coli or other micro organisms, but also food grade integration vectors called Ori^+ vectors have been developed. Ori^+ vectors only contain the replication origin of the *Lactococcus lactis* plasmid pWV01 which only replicates when the RepA replication protein is present. *Escherichia coli* and *Lactococcus lactis* strains were constructed with the *repA* gene in trans in their chromosomes supporting replication of the Ori^+ integration vectors (Leenhouts et al. 1996).

An integrative vector that allowed the selection of stable mutants capable of expressing foreign genes under the tight control of the *lac* operon promoter has been developed for the regulation of gene expression in *Lactobacillus casei* (Gosalbes et al. 2000). The lactose operon from *Lactobacillus casei* is regulated by glucose repression and lactose induction mechanism. The integrative expression vector contains two regions of homology that are physically close in the *Lactobacillus casei* chromosome, allowing stable gene insertion into the chromosomal lactose operon by homologous recombination.

Two other expression systems based on threonine- and pyrimidine auxotroph derivative *Lactococcus lactis* strains allow heterologous protein expression (Sørensen et al. 2000). For the pyrimidine auxotroph based system a cloning vector using an amber suppressor, *supD*, as selectable marker has been developed. *supD* encodes an altered tRNA and suppresses only amber codons. As host for this cloning vector, pyrimidine auxotrophs were generated by introduction of an amber codon into the chromosomal pyrF gene. This system allows selection and maintenance of the vector in pyrimidine free medium.

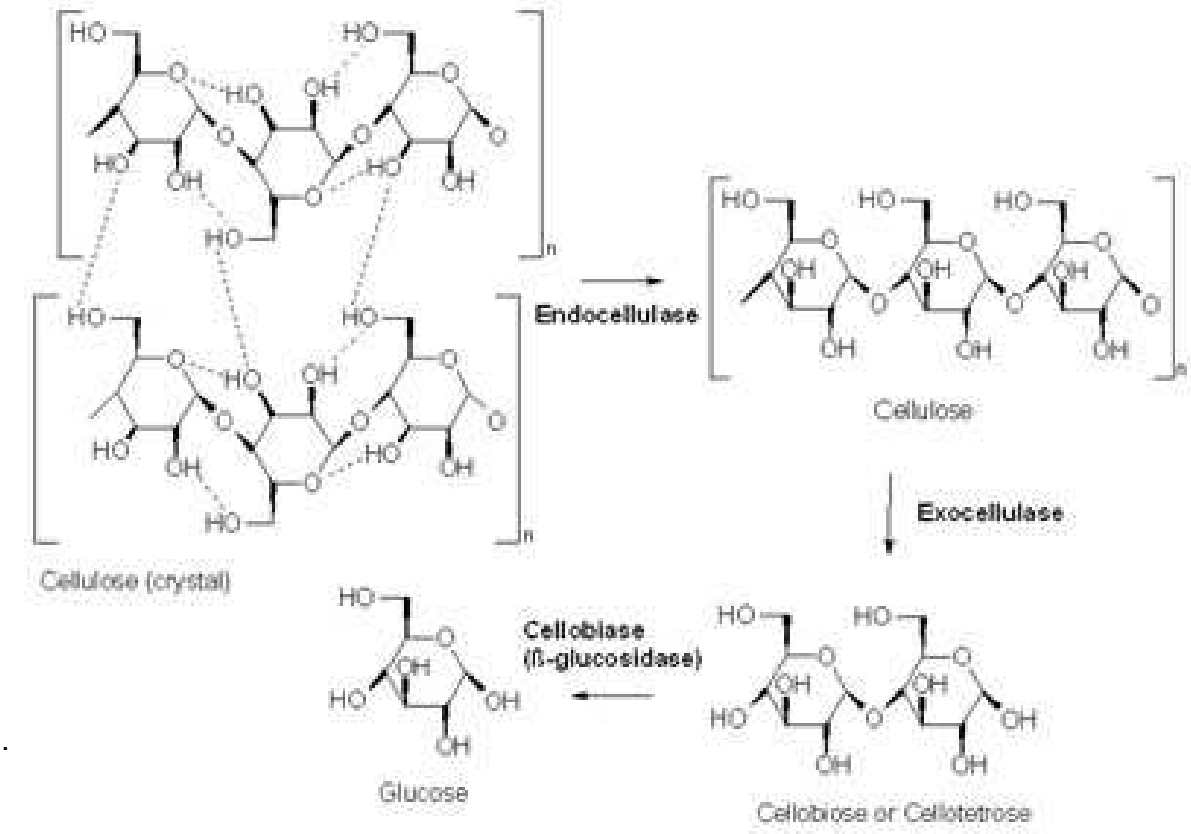
Other ingenious systems have been developed using a phage integrase-mediated site-specific insertion into the host chromosome (Alvarez et al. 1998) or stable integration in target genes, such as *cbh*, which encodes a bile salt hydrolase (Leer et al. 1993) via homologous recombination.

1.6 Degradation of cellulose

Cellulose is a carbohydrate polymer and a main component of the cell wall of plants. It predominantly occurs in long water-insoluble, crystalline microfibrils. These microfibrils each consists of several parallel oriented linear chains of (1→4) β-linked D-glucose and are highly resistant to enzymatic hydrolysis.

To exploit the energy stored in cellulose, organisms such as fungi and bacteria produce mixtures of synergistically acting cellulases (Béguin & Aubert 1994). Cellulases have a catalytic domain, linked to a cellulose binding domain by a glycosylated, Pro/Thr/Ser-rich peptide (Gilkes et al. 1991). The cellulose binding domain determines the efficiency of degradation of insoluble cellulose (Klyosov 1990).

There are two classes of cellulase systems. Non-complexed cellulase systems consist of a set of three soluble enzymes. The Endo-cellulase breaks internal bonds to disrupt the crystalline structure of cellulose and produces thereby short polysaccharide chains and small amounts of glucose monomers. The Exo-cellulase cleaves 2-4 units from the ends of the exposed chains produced by the endo-cellulase, resulting in disaccharides such as cellobiose. Cellobiase or beta-glucosidase hydrolyses the β-glycosidic linkage between the two glucose molecules in the cellobiose leading to its constituent glucose monomers. Noncomplexed cellulase systems are typical of cellulose-degrading aerobic fungi.
Complexed cellulase systems are composed of the same three enzyme types, but they are bound together forming a multi-protein complex. Complexed cellulases are typically found in anaerobic cellulose-degrading bacteria.



.

Figure 1.4: The three types of reaction catalyzed by cellulases:1. endo-cellulase: breakage of the non-covalent interactions present in the crystalline structure of cellulose. 2. exo-cellulase: hydrolysis of the individual cellulose fibers to break it into smaller sugars. 3. Cellobiase: hydrolysis of disaccharides into glucose monomers. (Figure from en.wikipedia.org/wiki/Image:Types_of_Cellulase2.png; Dec. 2008)

2 Objectives

Lactic acid bacteria play an important role in the preservation of ensiled forages from microbial and enzymatic deterioration. The efficiency of preservation depends on the rapidity of acidification by lactic fermentation. The rate of acidification is constrained by the concentration of water-soluble carbohydrates in the row plant material as the limiting factor for lactic acid bacterial growth.

The aims of this work were:

1. Construction of a transfer vector permitting vector free cloning
2. To establish an optimized transformation protocol for a commercial *L. plantarum* strain.
3. To express an endo-1,4-β-glucanase gene in *L. plantarum* to answer the question if the acidification performance of the recombinant strain *L. plantarum* E-1,4-BG is better than the performance of the wild type strain.

3 Materials and Methods

3.1 Materials

3.1.1 Equipment

Table 3.1: Equipment

device	supplier
agarose gels unit	Midi Horizontal Gel Unit, SCIE-PLAS, (UK)
centrifuges	Avanti J-20-XP, Beckman Coulter (USA) 5415 D, Eppendorf (Germany) C312, Jouan (Germany)
chemiluminescence imager	Lumi-Imager F1, Boehringer Mannheim, GmbH (Germany)
class II laminar flow	Herasafe, Heraeus (Germany)
SDS-PAGE electrophoresis units	SE 250/SE 260, Hoefer (USA) Mini-Cell, Novex (USA)
gel caster	260 series, Hoefer (USA)
gel documentation system	Gel Doc 2000, Bio-Rad (USA)
incubators	B 115, WTB-Binder (Germany) BK 6160, Heraeus (Germany)
pH electrode	InLAB 417, Mettler-Toledo (Germany))
pH meter	pH 521, WTW (Germany)
pipette tips, centrifuge tubes	Greiner Bio-One (Germany)
power supply	Power Pac 200, Bio-Rad (USA)
scales	A-120-S, Sartorius (Germany) SM-L, Mettler-Toledo (Germany)
semi dry transfer cell	Trans-Blot SD, Bio-Rad (USA)
NanoPhotometer	Implen (Germany)
electroporators	MicroPulser, Bio-Rad (USA) ECM 630 Presision Pulse, BTX Harvard apparatus (USA)
transilluminator	TFB-M/WL, Vilber-Lourmat (France)

3.1.2 Reagents

All salts, alcohols, acids, bases and ingredients, for bacterial media, were obtained from Merck (Germany). Ultra pure Tris, DMSO, TEMED, EDTA, MRS agar and MRS bouillion were purchased from Roth (Germany). Tween 20, Triton X-100 and ethidiumbromide were obtained from Sigma (Germany). Agarose was purchased from Invitrogen (USA). Acrylamide:N,N'-methylene-bisacrylamide 37,5:1 solution was supplied by Fluka (Switzerland).

3.1.3 MEDIA

All media were prepared with deionized water and were sterilized by autoclaving at 120°C for 20 minutes.

3.1.3.1 Growth medium for *Escherichia coli* (*E. coli)* strains

- **LB medium (Luria-Bertani medium*)***

  10 g/l peptone
  5 g/l yeast extract
  10 g/l NaCl
  Components were dissolved in water, pH was adjusted to 7.0.
  After autoclaving, medium was stored at 16°C

- **LB agar**

  1.5% agar agar was dissolved in LB medium before autoclaving.
  After autoclaving, medium was stored at 16°C

- **SOC medium (super optimal catabolite medium)**

  20 g/l bacto tryptone
  5 g/l yeast extract
  10 mM NaCl
  3 mM KCl
  10 mM $MgCl_2$

10 mM $MgSO_4$

20 mM glucose

Components were dissolved in water, pH was adjusted to 7.0.

After autoclaving, medium was stored at 4°C

3.1.3.2 Growth medium for *Lactobacillus plantarum (L. plantarum*)

- **MRS bouillion**

  10 g/l peptone

  4 g/l yeast extract

  8 g/l meat extract

  20 g/l glucose

  2 g/l K_2HPO_4

  5 g/l sodium acetate

  2 g/l ammonium citrate

  0.2 g/l $MgSO_4$

  0.05 g/l $MnSO_4$

  1.0 g/l Tween 80

  Components were dissolved in water, pH was adjusted to 6.2.

  After autoclaving, medium was stored at 16°C

- **MRS agar**

  1% agar agar was dissolved in MRS bouillion before autoclaving.

  After autoclaving, medium was stored at 16°C

- **MRS agar + 2 % Cellulose**

  10 g cellulose (Cellulose powder CF11, Whatman, England) were dissolved in 500 ml MRS agar before autoclaving.

3.1.4 DNA markers & loading dyes

All DNA markers and loading dyes were purchased from Fermentas (Germany) or from New England Biolabs.

6x DNA Loading Dye
GeneRuler™ 1 kb DNA Ladder
GeneRuler™ DNA Ladder Mix
NEB, TriDye 2 log Ladder

3.1.5 Protein markers

Protein marker was purchased from Fermentas.

PageRuler™ Prestained Protein Ladder

3.1.6 Antibiotics

Ampicillin and Chloramphenicol were purchased from Roth. Erythromycin was purchased from Fluka.

Ampicillin
stock solution: 100 mg/ml (in ddH_2O)

Chloramphenicol
stock solution: 25mg/ml (in EtOH)

Erythromycin
stock solution: 10 mg/ml (in ddH_2O)

3.1.7 Enzymes

All restriction enzymes as well as the calf intestine alkaline phosphatase (CIAP) and T4-ligase including appropriate 10x buffers, were purchased from New England Biolabs.
KOD HiFi and XL DNA polymerase were purchased from Novagen. Taq polymerase, used only for colony PCR, was purchased from BioTools.

3.1.8 PLASMIDS

- **pU19 vector**

  pUC19 vector was purchased from Invitrogen.

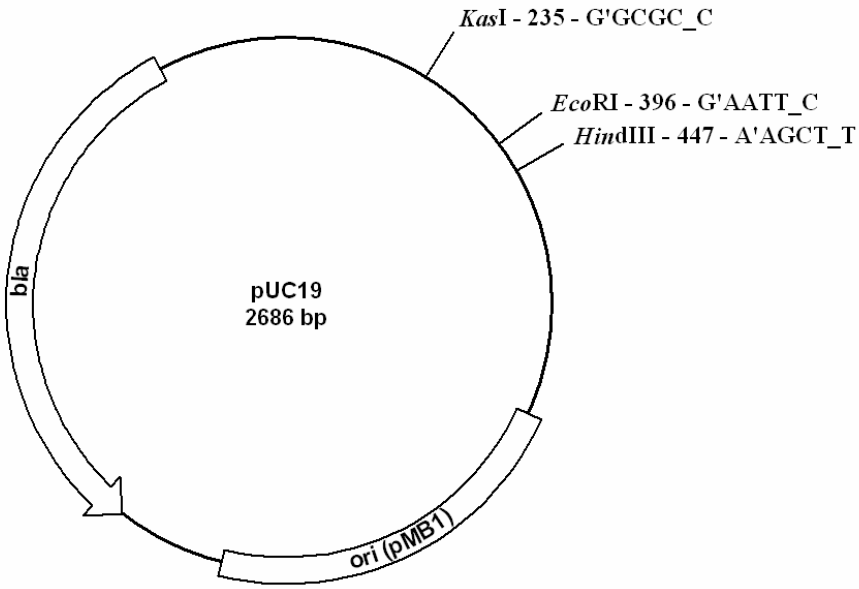


Figure 3.1: Figure shows a simplified map of the pUC19 vector. An origin sequence (ori (pMB1)) permits plasmid amplification and an ampicillin resistance gene (bla) permits selection of transformants in *E. coli.* Relevant restriction sites are indicated.

3.1.9 PRIMERS

Restriction sites are highlighted in grey.

Primers for endo-1,4-beta glucanase amplification

cellulase *Sac*I_back
5‘-GATGATGAGCTCAAACGCTGAAACATGGGAAG-3‘

cellulase *Sac*I_for
5‘-GATGATGAGCTCAATCTTCCAGCGTCATATCG-3‘

cellulase *Kpn*I back
5’-ACTACTGGTACCATGAAACGGTCAATCTCTATTTTTATTAC-3’

cellulase *Sal*I for
5’-ACTACTGTCGACTAATTTGGTTCTGTTCCCCAAATCAG-3’

Primers for Cellulase HisTag amplification

His-tag cellulase KpnI_for
5’-ATGATGGGTACCAATTAGTGATGGTGATGGTGGTGATTTGGTT
CTGTTCCCCAAAT-3’

Screening primers for genom integration

plantarum 3’-cbh
5’-AACTGATAACAAACTGATTTCC-3’

plantarum 5’-cbh
5’-TCTAAATATTATTTTCGAGGAGG-3’

Pimers for resistance gene amplification

ery_*Kas*I_back:
5‘-CATCATGGCGCCTCCGATTGCAGTATAAATTTAACG-3‘

ery_*Kas*I_for:
5‘-CATCATGGCGCCAACTCGGCGTATGTTATTCAAG-3‘

cat_*Kas*I_back:
5‘-CATCATGGCGCCCTCGCCGGCAATAGTTACC-3‘

cat_*Kas*I_for:
5‘-CATCATGGCGCCAATGTCGGCATAGCGTGAGC-3‘

CAT_*Sph*I_back (Tn7):
5‘-GATGATGCATGCTACGGGGAGAGCCTGAGC-3‘

CAT_*Sph*I_for (Tn7):
5‘-GATGATGCATGCGGAGCACCTCAAAAACACC-3‘

Primers for construction of multiple cloning site (MCS)

MCS_pUC19_back
5‘-GCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGC
ATGCA-3‘

MCS_pUC19_for
5‘-CTAGTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTACC
GAGCTCG-3‘

Primers for ori pE194 amplification

pE194_720_*Kas*I
5‘-ACTCATGGCGCCTTTCGGTTGCAAAGCTCTAGG-3‘

pE194_720_*Hind*III
5‘-ACTCATAAGCTTTTTCGGTTGCAAAGCTCTAGG-3‘

pE194_1979_*Hind*III
5‘-ACTCATAAGCTTCTTAACTTGTTTTTCGTGTGCC-3‘

Pimers for cbh amplification

cbh_c_*Hind*III_for
5‘-GATGATAAGCTTAGTTAACTGCATAGTATTGTGC-3‘

cbh_n_*Eco*RI_back
5‘-GATGATGAATTCATGTGTACTGCCATAACTTATC-3‘

cbh_n_*Sac*I_for
5‘-GATGATGAGCTCGATTGTCGTAAATTTTTAGTCC-3‘

cbh_c_*Sph*I_back
5‘-GATGATGCATGCAAACAATCCTAATTTTGACTACC-3‘

Primers for colony PCR

pUC19_back:
5‘-TGTCGGGGCTGGCTTAAC-3‘

pUC19_for:
5‘-AGTTGCGCAGCCTGAATGG-3‘

M13
5‘-TGT AAA ACG ACG GCC AGT-3‘

M13R
5‘-CAG GAA ACA GCT ATG ACC-3‘

CATseq_back
5‘-GTTATTGGGATAAGTTAGAGC-3‘

MCS_screen_back
5‘-TCGGTACCCGGGGATCC-3‘

MCS_screen_for
5‘-AGGATCCCCGGGTACCG-3‘

eryseq_back:
5‘-ATATTAAGTATGGTTCCAAGAG-3‘

Cellulase_n_n_for
5’-ACGTAATAAAAATAGAGATTGACC-3’

Cel_seq_back
5‘-ACCAGCTAAAAGATGCAAACG-3‘

Cel_seq_for
5‘-TTGCTGAGTGCATAGTTTGC-3‘

ori_pE194_seq_for
5‘-ATGTTTTCTCCCCTAAATAACC-3‘

CAT_seq2_back
5‘-TACATCATTCTGTTTGTGATGG-3‘

3.1.10 MICROORGANISMS

- ***E. coli* strains**

  Strain DH10B, purchased from Invitrogen

  Genotype: F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80dlacZΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara leu)7697 galU galK rpsL endA1 nupG

- ***L. plantarum* strains**

  L. plantarum 268 DSM 12836

- ***B. subtilis* strains**

  B. subtilis. subsp. *subtilis*, strain SB202 (plasmid pC194) DSM 4393

  B. subtilis. subsp. *subtilis*, strain 1E18 (plasmid pE194) DSM 4554

  B. subtilis strain 168 DSM 4424

3.2 Methods

3.2.1 CULTIVATION OF MICROOGANISMS

- **Cultivation of *E. coli***

E. coli was grown over night on LB agar or in LB medium at 37°C with shaking at 180 rpm.

If required, media were supplemented with antibiotica.

- **Cultivation of *L. plantarum***

L. plantarum was grown for one to three days on MRS agar or in MRS bouillion at 30°C under oxygen limitation.

If required, media were supplemented with antibioticum or cellulose.

3.2.2 BIOMOLECULAR METHODS

All buffers and solutions were prepared with double distilled water (ddH_2O). For all enzyme reactions and PCRs sterile ddH_2O was used.

3.2.2.1 Preparation of plasmid DNA

Plasmid DNA was prepared using the preGOLD plasmid miniprep Kit II (peQLab, Germany) or the midiprep kit (Promega, Germany), according to the producer's recommendation.

3.2.2.2 Preparation of genomic DNA

Genomic DNA was isolated using the DNeasy blood and tissue extraction kit (Qiagen, Germany), according to the producer´s protocol for Gram positive bacteria.

3.2.2.3 Preparation of *E. coli* for electroporation

20 ml LB medium were inoculated with the appropriate *E. coli* strain and incubated over night at 37°C with shaking. Two 2000 ml Erlenmeyer flasks, filled with 400 ml LB medium, were inoculated 1:100 with the prepared over night culture and incubated at 37°C with shaking until an OD_{600} of 0.6 was reached. Bacteria were harvested by centrifugation at 10000 rcf for 8 minutes at 4°C.The pellets were washed with 500 ml, 250 ml, 100 ml ice cold 1 mM HEPES and finally with 30 ml 10% glycerol. The pellet was resuspended in 3 ml 10% glycerol. 50 µl aliquots of the cell suspension were frozen in liquid nitrogen immediately and stored at -80°C until further use.

3.2.2.4 Preparation of *L. plantarum* for electroporation

25 ml MRS bouillion were inoculated with the *L. plantarum* strain 268 and incubated over night at 30°C under oxygen limitation. The prepared over night culture was diluted with MRS bouillion + 1% glycin to OD_{600} of 0.2 and

incubated at 30°C under oxygen limitation until an OD_{600} of 0.6 was reached. The cells were harvested by centrifugation at 10000 rcf for 5 minutes at 4°C. The pellets were washed twice with ice cold sterile H_2O and once with 30% PEG_{1500} or with 952 mM sucrose with 3.5 mM $MgCl_2$. The pellet was resuspended to one hundredth of initial volume in 30% PEG_{1500} or in 952 mM sucrose with 3.5 mM $MgCl_2$ and kept on ice until electroporation.

3.2.2.5 Polymerase Chain Reaction (PCR)

The polymerase chain reaction is a method for DNA amplification. DNA synthesis is performed by a thermo stable DNA polymerase. All PCRs were carried out with a T3 Thermocycler (Biometra, Germany).

- **PCR amplification of inserts**

  Amplification of DNA inserts was performed using the proofreading KOD DNA polymerase and genomic DNA as a template. The PCR master mix is stated in table 3.2.

  KOD standard protocol

Table 3.2: PCR master mix used for amplification of DNA inserts

50 µl PCR master mix:	volume
KOD buffer #1 (10 x)	5.0 µl
dNTPs (2 mM each)	5.0 µl
$MgCl_2$ (25 mM)	3.0 µl
template DNA (0.006-6ng)	1.0 µl
KOD DNA-polymerase (2.5 U/µl)	0.4 µl
sense primer (10 pmol/µl)	2.0 µl
anti-sense primer (10 pmol/µl)	2.0 µl
sterile ddH_2O	to 50.0 µl

Thermocycler program

98°C	35 sec	
Tm-5°C	15 sec	30 cycles
72°C	25 sec/2kbp	
72°C	5 min	

- **PCR screening for positive *E. coli* transformants**

  Taq DNA polymerase was used for screening positive *E. coli* transformants by colony PCR (with DMSO and RNase added). A single colony was picked with a pipette tip and suspended in 35 µl PCR master mix in a PCR tube.

Taq polymerase standard protocol

Table 3.3: PCR master mix used for Taq polymerase standard PCR

100 µl PCR master mix:	volume
10 x buffer	10.0 µl
Taq polymerase (5U/µl)	0.5 µl
DMSO (optional)	6.0 µl
RNase A (100mg/ml, optional)	0.2 µl
dNTPs (10 mM each)	1.0 µl
$MgCl_2$ (50 mM)	4.0 µl
sense-primer (10 pmol/µl)	1.5 µl
antisense-primer (10 pmol/µl)	1.5 µl
sterile ddH_2O	to 100.0 µl

Thermocycler program

94°C	5 min	
94°C	30 sec	
53°C	30 sec	30 cycles
72°C	1 min/kbp	
72°C	5 min	

- **Screening for positive *L. plantarum* transformants by colony PCR**

  A single colony was picked with a pipette tip and suspended in 10 µl ddH_2O. The bacterial suspension was denaturated at 99°C for 5 minutes. Then 35 µl KOD standard PCR master mix (see table 3.2) were added. PCR was performed according to the KOD standard protocol (see section 3.2.2.5).

- **PCR screening for *L. plantarum* integrants**

  Genomic DNA was prepared as described in section 3.2.2.2. 2 µl genomic DNA was suspended in 40 µl PCR mix A (see table 3.4.) in a PCR tube. The suspension was denaturated at 99°C for 5 minutes. Then 10 µl of PCR mix B (see table 3.4.) was added.

Table 3.4: PCR mix A and B used for screening *L. plantarum* integrants

mix A		**mix B**	
KOD buffer #1 (10 x)	4.0 µl	KOD DNA-polymerase (2.5 U/µl)	3.3 µl
$MgCl_2$ (25 mM)	2.0 µl	KOD buffer #1 (10 x)	10.0 µl
template (genomic DNA)	2.0 µl	dNTPs (2 mM each)	42.0 µl
sense primer (10 pmol/µl)	2.0 µl	sterile ddH_2O	to 100.0 µl
anti-sense primer (10 pmol/µl)	2.0 µl		
sterile ddH_2O	to 40.0 µl		

Thermocycler program

99°C	5 min	
90°C	1 min → During this step PCR mix B was added.	
98°C	35 sec	30 cycles
53°C	15 sec	
72°C	30 sec/ kbp	
72°C	5 min	

3.2.2.6 Purification of DNA fragments

DNA fragments were purified using the GFX Kit (GE Healthcare, UK) according to the producer's recommendation.

3.2.2.7 DNA extraction from gel bands

DNA from digested and dephosphorylated plasmids was gel-purified using the DNeasy gel extraction kit (Qiagen, Germany), according to the producer's recommendation.

3.2.2.8 Enzyme digestion

10 units of the required restriction enzyme were used per 1 µg DNA with the buffer according to the manufacturer's recommendations. The volume of the enzyme never exceeded 10% of the total volume. Incubation took place at optimal temperature for at least 90 minutes.

3.2.2.9 Dephosphorylation reaction

To prevent religation after enzyme digestion, the plasmids were dephosphorylated by adding 1 µl phosphatase and 2 µl of 10x phosphatase buffers directly to the 20 µl restriction reaction mix. The mix was incubated for further 30 minutes at 37°C.

3.2.2.10 Ligation reaction

For a 20 µl ligation reaction digested, dephosphorylated and gel-purified plasmid DNA was mixed on ice with 1 µl T4 DNA Ligase, 2 µl of 10x T4 ligase buffer and with a 5-fold molar excess of digested and purified insert DNA. The ligation reaction was incubated at 16°C over night or for 1 hour at room temperature.

3.2.2.11 Isopropanol precipitation

For DNA precipitation the same volume of isopropanol and 0.1 volumes of 3M ammonium acetate were added to the ligation. After an incubation of 15 min at room temperature the DNA was centrifuged at 16000 rcf for 15 min. The supernatant was discarded and the DNA pellet was resuspended in 2.5 volumes of 70% ethanol and again centrifuged at 16000 rcf for 15 min. The supernatant was discarded and the DNA pellet was air-dried for 15 min at room temperature. The pellet was dissolved in 15 µl ddH_2O and stored at 4°C until transformation.

3.2.2.12 Transformation of *E. coli* by electroporation

A 50 µl aliquot of electrocompetent cells was thawed on ice, mixed with 7 µl DNA solution from isopopanol precipitation and transferred to an ice cold electroporation cuvette with a 2 mm gap. Bacteria were electroporated using a BioRad MicroPulser. The electroporation conditions were 2.5 kV, 25 µF and 1000 Ω. Pulses with time constants ranging from 5.0 to 5.8 ms resulted in successful transformation. After electroporation, cells were revitalized for 30 minutes in 450 µl SOC medium at 37°C with shaking. After these 30 minutes cells were transferred on LB agar plates containing 100 µg/ml ampicillin for selection. Plates were incubated at 37°C over night.

3.2.2.13 Transformation of *L. plantarum* by electroporation

Before electroporation, the appropriate amount of plasmid DNA was added to 40 µl cell suspension. After incubation on ice for 10 minutes, the mixture was transferred to ice-cold electroporation cuvettes (0.2 cm gap, Sigma Aldrich, Germany) and elelctroporated using an ECM 630 Precision Pulse. The results were obtained using a constant capacitance setting of 25 µF and different voltages and puls control resistances. After electroporation, cells were revitalized in 340 µl MRS bouillon for 2 hours at 30°C. Then, cells were

transferred on MRS agar plates containing 5 µg/ml chloramphenicol for selection. Plates were incubated for 3 days at 30°C under oxygen limitation.

3.2.2.14 Agarose gel electrophoresis

To separate and quantify DNA fragments an agarose gel electrophoresis was performed. Samples were mixed with 6x BX buffer (0.25% bromphenol blue, 0.25% xylen cyanol FF, 30% glycerol) and applied to a 1% agarose gel (1% agarose in 1x TAE buffer). Also 6 µl of DNA length standard was applied. As electrophoresis buffer 1x TAE (40 mM Tris-acetate, 100 mM EDTA, pH 8.3) was used. Electrophoresis for analytical gels was performed at 130 V and for preparative gels at 110 V for 30 – 40 minutes.

3.2.2.15 Preparation of cryostocks

600 µl over night culture were diluted 1:2 with 40% glycerol and stored at -80°C.

3.2.2.16 Photometric determination of DNA concentration and cell density (OD_{600})

Photometric measurement was applied using the NanoPhotometer. DNA concentration was estimated at 260:280 nm. Cell density was estimated at 600 nm using polystyrene semi-micro cuvettes 10 x 4 x 45 mm (Sarstedt, Germany).

3.2.3 BIOCHEMICAL METHODS

3.2.3.1 SDS-PAGE (sodiumdodecylsulfate polyacrylamide gel electrophoresis)

Sodiumdodecylsulfate polyacrylamide gel electrophoresis is a standard method to separate proteins according to their molecular weight. SDS-PAGE was performed according to Laemmli (Laemmli 1970). The gel consists of the stacking gel and the resolving gel. First the resolving gel was cast and overlaid with 200 µl isopropanol. After the gel was polymerised the isopopanol was discarded. The stacking gel was cast directly on top of the resolving gel and combs were inserted immediately. When the stacking gel was polymerised, the gel chamber was transferred to the gel electrophoresis unit witch was filled up with Laemmli buffer (25 mM Tris, 250 mM glycine, 0.1% SDS w/v in ddH_2O) and the combs were removed. Before the samples were loaded on the gel, they were diluted with 2x loading buffer (10% SDS, 20% glycerol, 2% bromo phenol blue, 10% β-mercaptoethanol, 125 mM Tris-HCl pH 6.8 in ddH_2O) and heated to 99°C for 10 minutes. After the samples were loaded into each well, electrophoresis was performed at 200 V for 50 minutes.

Table 3.5: Compounds of SDS-PAGE gels

10% resolving gel (30 ml):	
15 ml	ddH_2O
7.5 ml	40% acrylamide
7.5 ml	1.5M Tris-HCl, pH 8.8
150 µl	20% w/v SDS
200 µl	10% w/v APS (ammonium persulfate)
40 µl	TEMED

stacking gel (4 ml):	
4.0 ml	stacking gel premix
34 µl	10% w/v APS
8 µl	TEMED

stacking gel premix (40 ml):	
24.6 ml	ddH_2O
10.0 ml	0.5 M Tris-HCl, pH6.8
5.0 ml	40% acrylamide
200 µl	10% w/v SDS

3.2.3.2 Silver stain

The gel was immobilised for one hour or overnight with fixation solution (50% v/v ethanol, 10% v/v acetic acid). After that the gel was incubated for 15 minutes in 25 ml sensitizing solution (30% v/v ethanol, 0.2% w/v $Na_2S_2O_3$, 7% w/v sodium acetate, 0.25% glutaraldehyde) and then washed three times for 5 minutes with ddH_2O. The gel was stained in 25 ml staining solution (0.05% w/v $AgNO_3$, 0.007% formaldehyde) for 10 minutes. Then it was washed once again with ddH_2O and treated with 25 ml developing solution (2.5% w/v Na_2CO_3, 0.007% formaldehyde) until bands were visible. To stop the staining reaction the gel was incubated with stopping solution (0.05 M Na-EDTA).

3.2.3.3 Western blot

Western blot is an immunodetection method for proteins. After SDS-PAGE the proteins are transferred to a membrane where they are probed using antibodies specific to the target protein.

After electroporation the gel and 4 pieces of 3M filter paper were equibrilated in Towbin buffer (192 mM glycine, 20% methanol, 25 mM Tris). The PVDF membrane was activated for 1 minute in methanol. For the blotting procedure two filter papers, membrane, gel and again two filter papers were put one upon the other on a Semi-Dry Blotter (Biorad). The transfer of the protein from the gel to the membrane was performed at constant voltage of 120 mA for 40 minutes. Afterwards the membrane was blocked in TPBS (20 mM sodium phosphate buffer pH 7.4, 150 mM sodium chloride, 0.1% Tween 20) containing 3% BSA (bovine serum albumin) for 1 hour at room temperature or over night at 4°C. After blocking, the membrane was washed with TPBS and incubated with α-tetra-His antibody (Qiagen, Germany) diluted (1:1000) in TPBS containing 1% BSA for 1 hour. The membrane was washed three times for 5 minutes with TPBS and then incubated with an anti mouse IgG-alkaline phosphatase conjugate (Sigma) diluted (1:2500) in TPBS containing 1% BSA. After 1 hour the membrane was washed again three times for 5 minutes with TPBS. Detection was performed by addititon of CDP-Star reagent (New England Biolabs, USA) as substrate for the

alkaline phosphatase according to manufacture´s recommendation. Luminescence was measured using the Lumi Imager.

3.2.3.4 Congo red assay

For screening the cellulase activity, transformed cells were plated on MRS agar with 2% cellulose and cultivated for three days at 30°C. 10 ml Congo-red solution (0.2% Congo-red, 25mM Tris/HCl pH8) was added to the plates and plates were shaked gently for 30 minutes. The Congo-red solution was discarded and the plates were washed three times with wash solution (0.5 M NaCl, 25 mM Tris/HCl pH 8) for 5 minutes. Zones of cellulose hydrolysis around colonies were decolourize by washing, leaving a yellow halo against a red background.

4 Results and discussion

4.1 Construction of the transfer vector

One aim was to design a transfer vector permitting vector-free cloning in *L. plantarum*. Therefore, a vector was constructed containing an antibiotic resistance gene for selection in *L. plantarum* and a multiple cloning site (MCS) for easy replacement of different target genes flanked by regions homologous to the appropriate locus of integration.
The transfer vector was constructed based on the pUC19 vector. First the non essentional cbh gene (conjugated bile acid hydrolase) form *L. plantarum* was amplified by KOD standard PCR using the primers cbh_c_*Hind*III_for/cbh_n_*Eco*RI_back and genomic DNA isolated from *L. plantarum* 268 served as template. The amplicon was double digested with *Eco*RI and *Hind*III and cloned into pUC19 vector yielding plasmid pU19_cbh. The plasmid was introduced into *E. coli* DH10B by electroporation. Ampicillin resistant transformants were screened by colony PCR. The plasmid was isolated by miniprep from the positive clones and sequence was verified by DNA sequence analysis.

The MCS site was constructed with the primers MCS_pUC19_back/MCS_pUC19_for and cloned into the plasmid pUC19_cbh which was digested with *Xba*I yielding plasmid pU19_cbh_MCS. The plasmid was again introduced into *E. coli* DH10B by electroporation and positive transformants were screened by colony PCR. The plasmid was isolated by miniprep from positive clones and sequence was verified by DNA sequence analysis.

The chloramphenicol resistance gene and the erythromycin resistance gene from pC194 or pE194 were amplified by KOD standard PCR using the primers cat_*Kas*I_back/cat_*Kas*I_for or ery_*Kas*I_back/ery_*Kas*I_for and genomic DNA isolated from *B. subtilis* DSM 4393 (for chloramphenicol resistance gene) and from *B. subtilis*

DSM 4554 (for erythromycin resistance gene) as templates. The amplicons were digested with *Kas*I and cloned into the plasmid pUC19_cbh_MCS yielding plamid cbh_MCS_CAT and plasmid cbh_MCS_ery. The plasmids were introduced into *E. coli* DH10B by electroporation and the ampicillin resistant transformants were screened by colony PCR. The plasmids were isolated by miniprep from the positive clones and sequence was verified by DNA sequence analysis

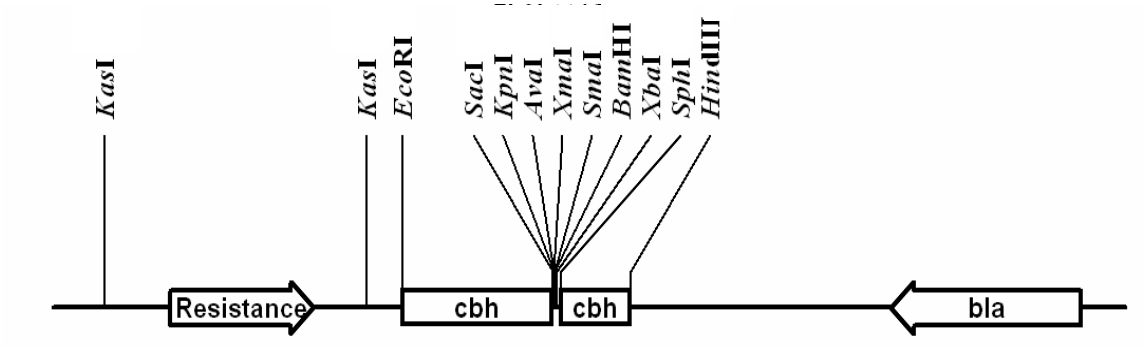


Figure 4.1: Linearized plasmid cbh_MCS and resistance gene (chloramphenicol/ erythromycin)

Maps of the designed vectors are shown in figures 3.2 and 3.3.

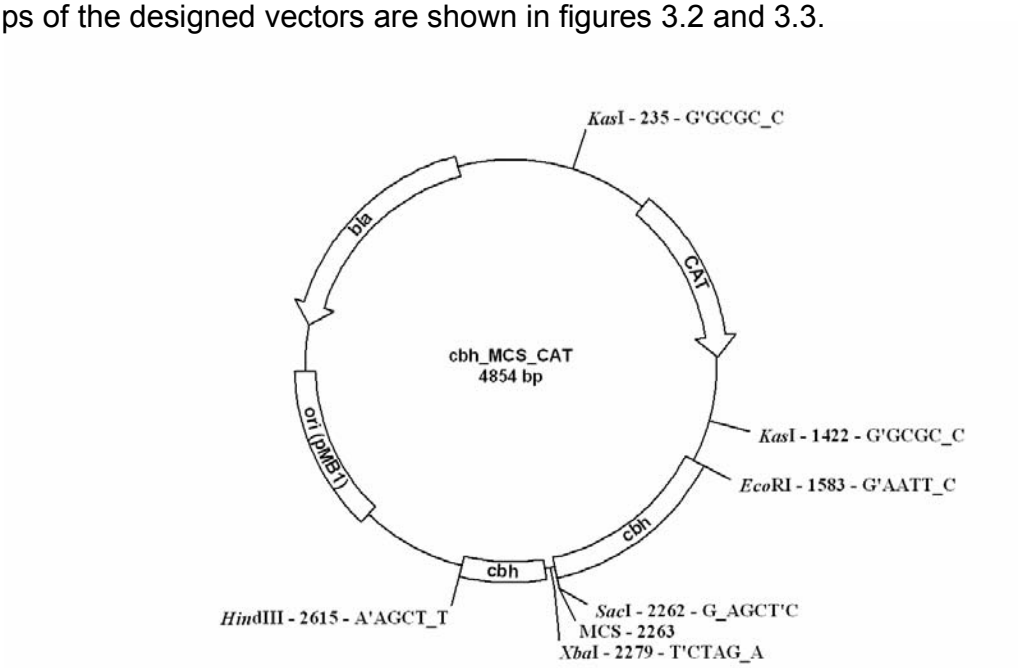


Figure 4.2: Map of the transfer vector cbh_MCS_CAT with a chloramphenicol resistance for selection of positive *L. plantarum* transformants.

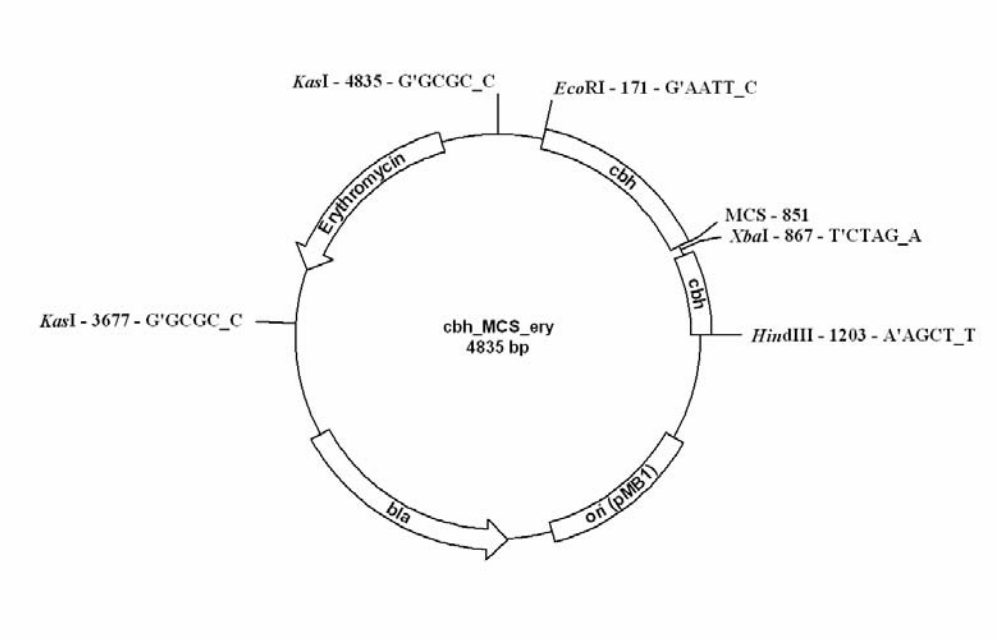


Figure 4.3: Map of the transfer vector cbh_MCS_ery with an erythromycin resistance for selection of positive *L. plantarum* transformants.

Transformation experiments using vector cbh_MCS_CAT in *L. plantarum* did not result in any colonies. With the vector cbh_MCS_ery in *L. plantarum* colonies were obtained, but it turned out that the colonies did not contain the plasmid. To look if the *L. plantarum* wild typ strain has an existing resistance to erythromycin, the strain was plated out on plates with an erythromycin concentration ranging from 5 to 50 µg/ml.

It turned out that the *L. plantarum* strain has an existing resistance to erythromycin because growth was detectable up to a concentration of 25 µg/ml erythromycin.

Because erythromycin is a preferable selection marker in many lactobacillus strains, further experiments with this vector were performed as well as with the vector cbh_MCS_CAT.

To raise the probability of a recombination event, the ori pE194 from *B. subtilis* was cloned into the vectors in order to obtain marginal plasmid replication, leading to a higher efficiency of integration.

The ori pE194 for the vector cbh_MCS_CAT was amplified by KOD standard PCR using the primers pE194_720_*Hind*III/pE194_1979_*Hind*III and genomic DNA isolated from *B. subtilis* 4554 as template. The amplicon was digested with *Hind*III and cloned into the plasmid. The ori pE194 for the vector cbh_MCS_ery was amplified by KOD standard PCR using the primers pE194_720_*Kas*I/ery_*Kas*I_back and genomic DNA isolated from *B. subtilis* 4554 as template. The amplicon was digested with *Kas*I and cloned into the plasmids described above yielding the plasmid cbh_MCS_CAT_pE194ori and plasmid cbh_MCS_pE194ori_ery_rev. The plasmids were introduced into *E. coli* DH10B by electroporation. Ampicillin resistant transformants were screened by colony PCR. The plasmids were isolated from positive clones by miniprep and sequence was verified by DNA sequence analysis.

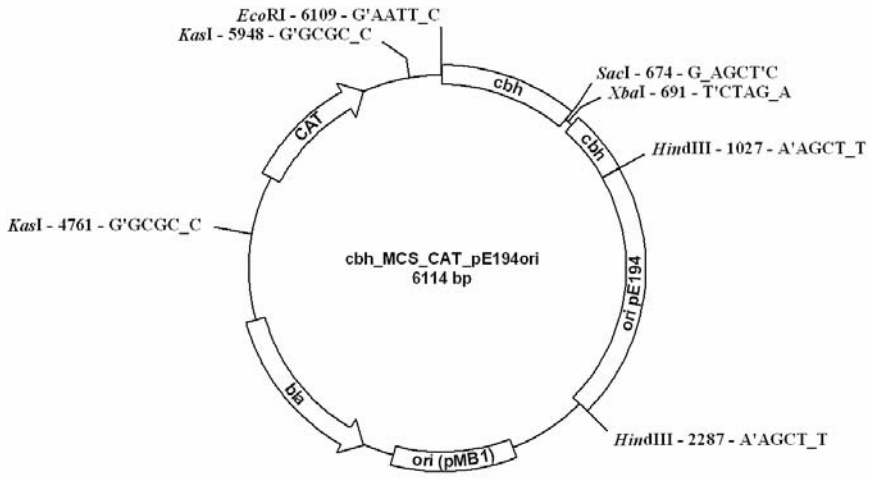


Figure 4.4: Map of the transfer vector with a chloramphenicol resistance and the ori pE194

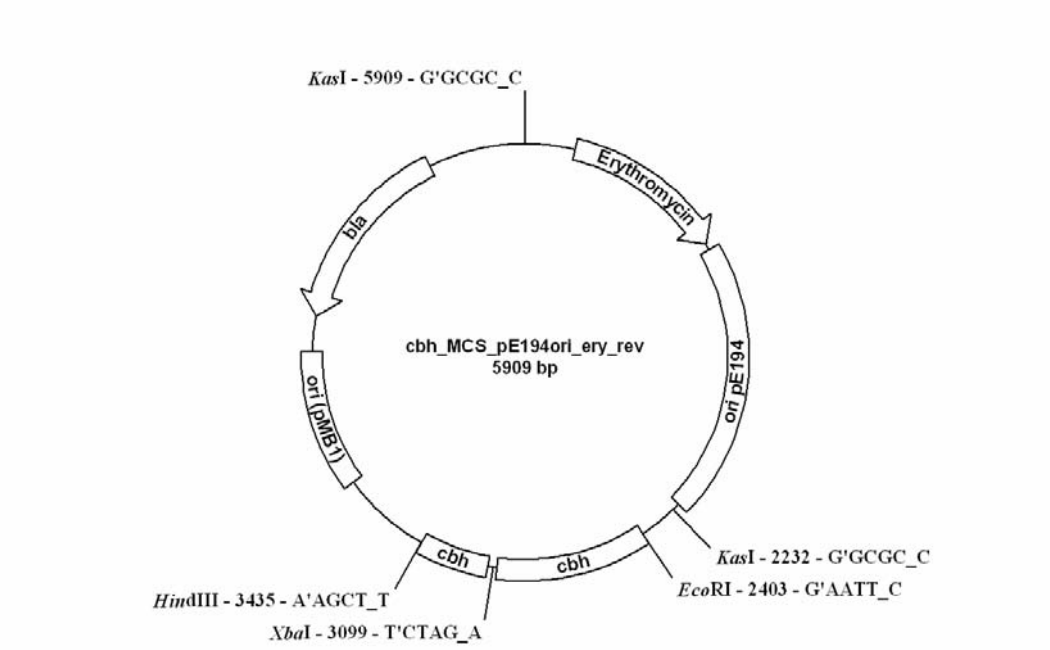


Figure 4.5: Map of the transfer vector with an erythromycin resistance and the ori pE194

Positives transfromants in *L. plantarum* were obtained with the vector cbh_MCS_CAT_pE194ori but not with the vector cbh_MCS_pE194ori_ery_rev. The erythromycin resistance was not usable as selection marker at the used concentrations because growth of negative *L. plantarum* transformants was detectable up to a concentration of 50 µg/ml erythromycin.

Further experiments were performed with the transfer vector cbh_MCS_CAT_pE194ori because selection of the *L. plantarum* transformants worked at a concentration of 5 µg/ml chloramphenicol.

4.2 Optimization of the transformation of *L. plantarum* 268

Several protocols for electrotransformation of different *Lactobacillus* strains have been published and it turned out that only selected strains could be transformed with high efficiency. But most LAB strains take up exogenous DNA at very low frequency. Because of strain variations, it was necessary to optimize the electroporation conditions for the *L. plantarum* 268 strain to obtain efficient genetic transfer.
All experiments were performed as triplicate and pU19 was taken as negative control.

4.2.1 Influence of electroporation solution on transformation efficiency

The first transformation experiments were based on the data from Aukrust and Blom (Aukrust & Blom 1992).
Cells were pepared for electroporation and transformed in 30% PEG_{1500} and 952 mM sucrose with 3.5 mM $MgCl_2$, respectively as described in section 3.2.2.4. The optimum conditions in PEG_{1500} solution were found to be 1.5 kV at 400 Ω and in sucrose-magnesium solution 1.5 kV at 800 Ω. Transformation was successful in PEG_{1500} at 1.5 kV, 400 Ω and 350 ng plasmid DNA.
Under these conditions with *L. plantarum* 268 no colonies were obtained. At higher plasmid concentration transformants were achieved but with a low transformation efficiency.

Table 4.1: Number of transformants after electroporation of *L. plantarum* 268 in 30% PEG_{1500}

voltage [kV]	1.5
resistance [Ω]	400
plasmid [ng]	1000
time const. [ms]	9.2
mean of transformants	55.3
standard deviation	18.61

Experiments were performed also in the electroporation solution sucrose-magnesium at 1.5 kV and 800 Ω. Transformation efficiency at these conditions was lower than in PEG_{1500} as shown in figure 4.6.

Table 4.2: Number of transformants after electroporation of *L. plantarum* 268 in 952 mM sucrose with 3.5 mM $MgCl_2$

voltage [kV]	1.5
resistance [Ω]	800
plasmid [ng]	1000
time const. [ms]	15.8
mean of transformants	5.7
standard deviation	0.58

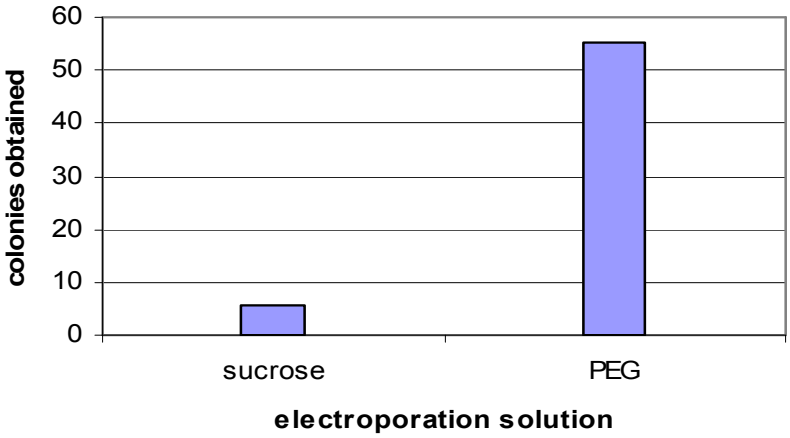


Figure 4.6: Number of transformants/µg DNA after electroporation of *L. plantarum* 268 strain, grown in MRS bouillion, 1% glycin using either 952 mM sucrose, 3.5 mM $MgCl_2$, 1.5 kV and 800 Ω or 30% PEG_{1500}, 1.5 kV and 400 Ω. Higher transformation efficiency could be reached using PEG as electroporation solution

Alegre et al. (Alegre et al. 2004) received good transformation efficiency in *L. plantarum* CECT 220 using sucrose as electroporation solution. In contrast to the results of Alegre et al., transformation of *L. plantarum* 268 was more efficient when PEG was used. Thus, the choice of the appropriated electroporation solution seems to be strain specific and has to be considered. In this case, PEG was used in further transformation experiments.

4.2.2 Influence of voltage and resistance on transformation efficiency

In order to optimize the electroporation conditions, factors of influence, plasmid concentration and time constant (t_c) which on its part depends on resistance and capacitance, were varied.

$$t_c = R \times C$$

t_c time constant
R resistance
C capacitance

1000 ng plasmid DNA were used for electroporation. Different voltages and different resistances were combined. A three dimensional plot was drawn to describe the effect on transformation efficiency. Results are shown in figure 4.7. Successful transformations were obtained at pulses with a time constant ranging from 4 to 6 ms. Also the combination of high voltage/low resistance and low voltage/high resistance turned out to be favourably for a successful transformation efficiency. The maximal colony numbers were reached at 2 kV and 200 Ω.

Table 4.3: Table shows number of transformantes obtained at different voltages at a constant resistance of 800 Ω.

voltage [kv]	resistance [Ω]	mean of transformants	standard deviation	time constante [ms]
1	800	4.3	6.66	17
1.5	800	41	11.53	16.6
2	800	0	0	12.7
2.5	800	0	0	---

Table 4.4: Table shows number of transformantes obtained at different voltages at a constant resistance of 400 Ω.

voltage [kv]	resistance [Ω]	mean of transformants	standard deviation	time constante [ms]
1	400	0	0	8.6
1.5	400	55.3	18.61	9.2
2	400	14	6.56	9.4
2.5	400	0	0	8.3

Table 4.5: Table shows number of transformantes obtained at different voltages at a constant resistance of 200 Ω.

voltage [kv]	resistance [Ω]	mean of transformants	standard deviation	time constante [ms]
1	200	1.6	1.5	4.5
1.5	200	56	7.94	4.4
2	200	59.3	11.02	4.6
2.5	200	9.3	5.13	4.7

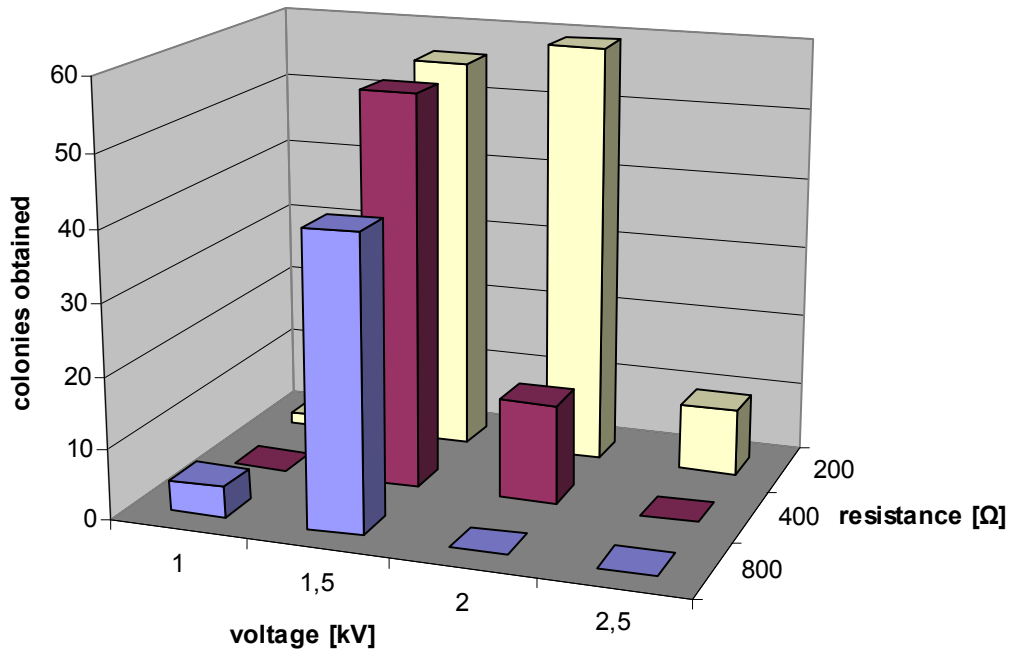


Figure 4.7: Transformation efficiency of *L. plantarum* 268 as a function of voltage and resistance, 30% PEG_{1500} was used as electroporation solution. Combination of high voltage/low resistance and low voltage/high resistance turned out to be favourably for successful transformation efficiency. The maximal colony numbers were reached at 2 kV and 200 Ω.

4.2.3 Influence of plasmid amount on transformation efficiency

Using optimized electroporation conditions, different plasmid concentrations were determined. As shown in the figure 4.8, the curve begins to flatten at a plasmid amount of 1000 ng and reached a plateau at a plasmid amount of 2000 ng. The number of tansformants begins to decrease with a higher plasmid amount than 2500 ng. As 1000 ng of DNA showed the best transformation efficiency, this plasmid amount was used in subsequent experiments.

Table 4.6: Table shows number of transformants obtained at different plasmid concentrations.

ng] plasmid DNA	transformants
100	26.3
350	54.6
1000	180
2000	247.3
2500	246.7

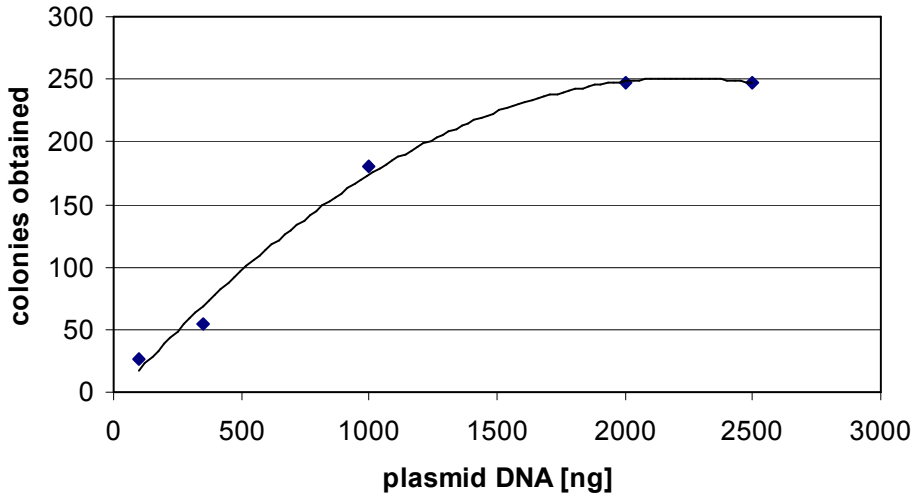


Figure 4.8: Effect of the plasmid DNA amount on the electrotransformation efficiency of *L. plantarum* 268 at 2 kV, 200 Ω. The curve begins to flatten at a plasmid amount of 1000 ng and reached a plateau at a plasmid amount of 2000 ng. The number of tansformantes begins to decrease with a higher plasmid amount than 2500 ng.

4.2.4 Influence of glycine concentration in the pre-incubation MRS bouillon

To further increase transformation efficiency different concentrations of glycine in the pre-incubation MRS bouillon were tested. The effect of increasing concentrations of glycine on growth and electro competence of *L. plantarum* is shown in table 4.7. The data indicate that a higher concentration of glycine in the medium inhibited the growth of the bacteria as indicated by the decreasing of the optical density of the culture. A concentration of 8% glycine resulted in growth stop. Also the number of transfromants did not increase with the concentration of glycine. The highest transformation frequencies were obtained, when glycine at a concentration of 1% was included in the growth medium, as previously reported by Aukrust and Blom (Aukrust & Blom 1992).

Table 4.7: Effect of glycine concentration in the pre-incubation medium on growth and electro competence of *L. plantarum*.

	OD600		
glycine concentration	**0h**	**3h**	**transformants**
1%	0.176	0.446	62.5
2%	0.178	0.295	3.0
8%	0.171	0.139	-----

Optimization of the transformation protocol of *L. plantarum* 268 showed that a transformation efficiency of $3x10^2$ transformants/µg DNA could be reached when the cells were grown in MRS bouillion +1% glycine and transformed under the following transformation conditions: electroporation solution: 30% PEG, plasmid amount: 1000 ng, capacitance: 25 µF, voltage: 2000 V, resistance: 200 Ω.

The higher transformation efficiency in *L. plantarum* CECT 220 reported by Alegre et al. (Alegre et al. 2004) (10^5 transformants/µg DNA) could result from the use of a different plasmid. The aim of this study was the integration of the plasmid into the host genome. Therefore, ori pE194 was used, leading to a marginal plasmid replication for efficient recombination.

4.3 Cloning and expression of an endo-1,4-β-glucanase gene form *B. subtilis* in *L. plantarum* 268

Goal was to introduce a cellulose degrading enzyme to improve the acidification performance of a commercial *L. plantarum* strain leading to a higher efficiency of preservation of ensiled forages.

4.3.1 Cloning of the endo-1,4-β-glucanase gene

The cellulase gene was amplified by KOD standard PCR using the primers cellulase *Sac*I_back/cellulase *Sac*I_for and genomic DNA isolated from *B. subtilis* DSM 4424 as template. The amplicon was digested with *Sac*I and cloned into the transfer vector cbh_MC_CAT_pE194ori yielding the plasmid cbh_MCS_CAT_pE194ori_cel. The plasmid was introduced into *E. coli* DH10B by electroporation. Ampicillin resistant transformants were screened by colony PCR. The plasmid was isolated by miniprep from positive clones and sequence was verified by DNA sequence analysis.

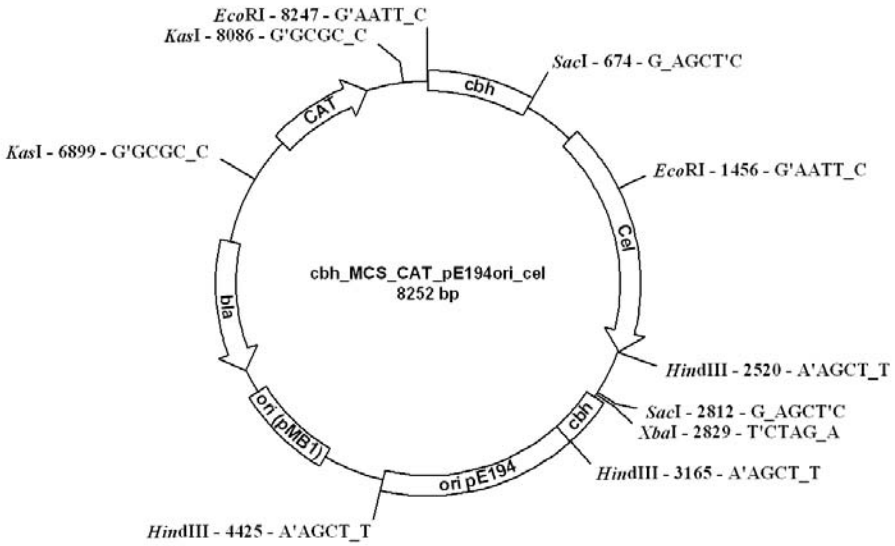


Figure 4.9: Map of the transfer vector cbh_MCS_CAT_pE194ori_cel

4.3.2 Genome integration by two-step homologous recombination

For the first step of homologous recombination of the transfer vector into the host chromosome, positive transformants were grown at 30°C for 72 hours in MRS bouillon supplemented with 5 µg/ml chloramphenicol. Dilutions of the cultures were plated onto MRS agar supplemented with 5 µg/ml chloramphenicol and incubated for 72 hours at 30°C. Integration of the vector was confirmed by PCR from genomic DNA using the primers plantarum 5'-cbh/Cellulase_n_n_for whereas one primer only binds to the chromosome of *L. plantarum* and the other binds specific to the integrated cellulase gene. The expected PCR fragment of 1100 bp was detected by agarose gel electrophoresis (see figure 4.11).

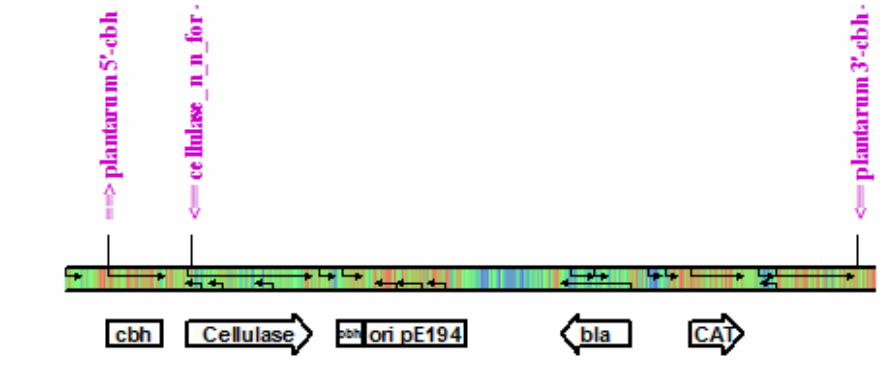


Figure 4.10: Map of the integrated vector in the chromosome of *L. plantarum* after the first step of homologous recombination.

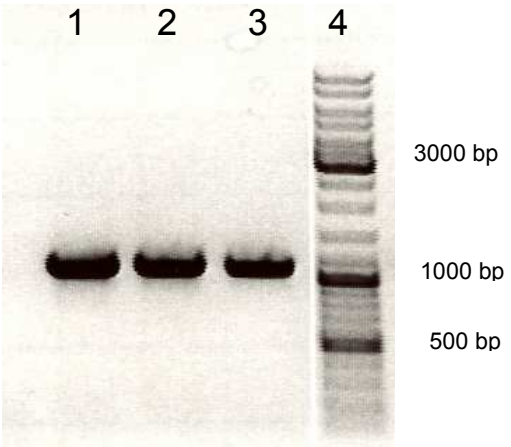


Figure 4.11: Colony PCR to confirm the chromosomal integration of the transfer vector. Lane 1-3: amplified integration fragment of positive clones. Lane 4: GeneRuler DNA Ladder Mix.

To remove the chloramphenicol resistance gene from the chromosome a second step of homologous recombination was induced by lowering the selective pressure in the growth medium. Clones with the vector integrated were grown in MRS bouillon without antibiotic for 72 hours at 30°C. Dilutions of the cultures were plated onto MRS agar without antibiotic. The second step of recombination was confirmed by PCR from genomic DNA using the primers plantarum 5'-cbh/plantarum 3'-cbh which only bind to the chromosome of *L. plantarum*. The expected PCR fragment of 3200 bp was detected by agarose gel electrophoresis (see figure 4.13).

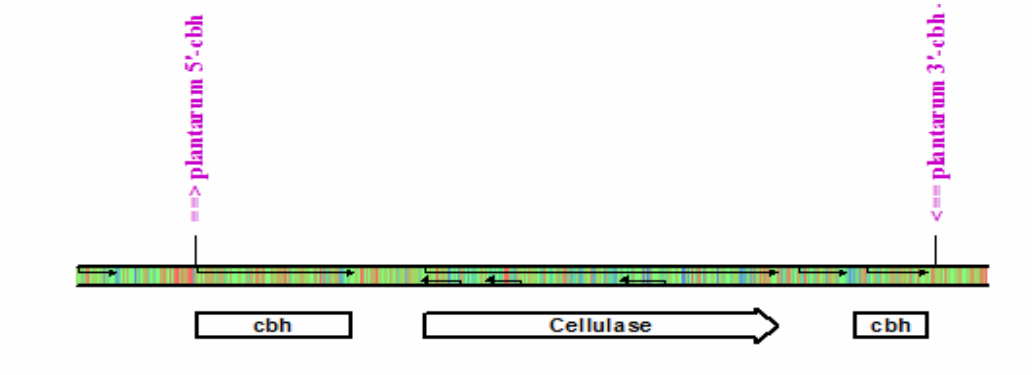


Figure 4.12: Map of the integrated vector in the chromosome of *L. plantarum* after the second step of homologous recombination.

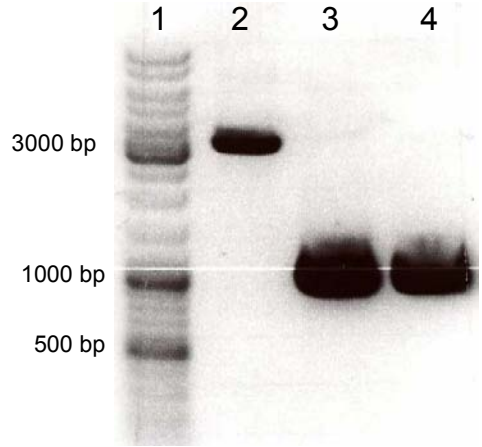
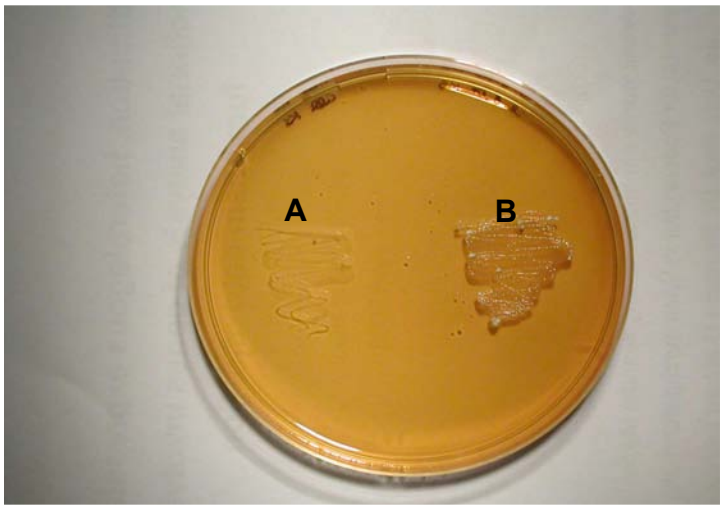


Figure 4.13: Colony PCR to confirm the second step of homologous recombination. Lane 1: GeneRuler DNA Ladder Mix. Lane 2: amplified fragment containing the target gene of a positive clone. Lane 3-4: negative control *L. plantarum* wild type strain.

Additionally, absence of the resistance gene was confirmed by plating a positive transfromant after the second step of homologous recombination onto MRS agar supplemented with 5 µg/ml chloramphenicol.

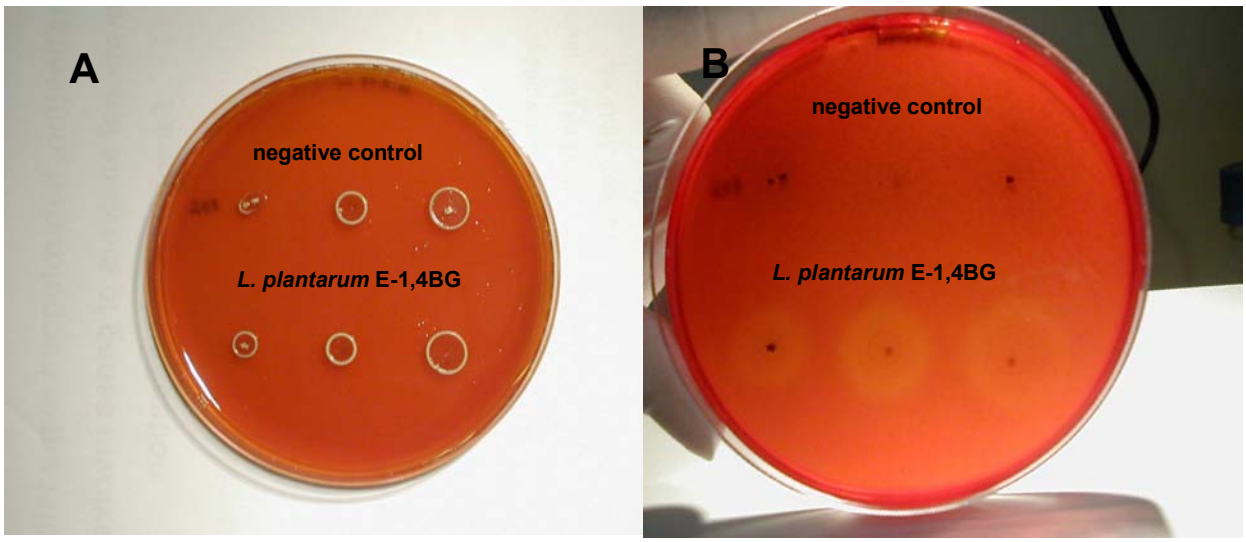


Picture 4.1: Growth of transformants on MRS agar plate supplemented with 5 µg/ml chloramphenicol. **A)** Transromant after the second step of homologous recombination. As a result of the absence of the resistance gene, no growth could be observed. **B)** Transformant after the first step of homologous recombination. Growth could be observed as a result of the existence of the resistance gene.

As can be seen from picture 4.1, bacterial growth could be detected after the first step of homologous recombination, but not after the second step of recombination as a result of the absence of the resistance gene.

4.3.3 Test of cellulase activity

Cellulase activity was detected by Congo red assay as described in section 3.2.3.4. MRS agar plates supplemented with 2% cellulose were inoculated with 2.5, 5 and 10 µl cell suspension (OD_{600}=1) each, of the recombinant *L. plantarum* strain E-1,4BG and the *L. plantarum* wild type strain as negative control (see picture 4.2).



Picture 4.2: Degradation of cellulose by the recombinant *L. plantarum* stain E-1,4-BG. **A)** Colonies prior to congo red assay. **B)** Halos indicate degradation of cellulose by *L. plantarum* E-1,4-BG.

Colonies of the recombinant *L. plantarum* E-1,4BG strain formed hydrolysis halos indicating cellulase activity. No degradation of cellulose was observed around colonies of the wild typ strain.

4.3.4 Expression of a his-tagged variant of the cellulase from *B. subtilis* in *L. plantarum*

For the detection of the cellulase on protein level by SDS-PAGE and Western Blot, a transfer vector was constructed with a his-tagged cellulase gene. Insert was amplified by KOD standard PCR using the primers cellulase *Sac*I_back/His-tag cellulase KpnI_for and genomic DNA isolated from *B. subtilis* DSM 4424 as template. The amplicon was double digested with *Sac*I and *Kpn*I and cloned into the transfer vector cbh_MC_CAT_pE194ori yielding the plasmid cbh_MCS_CAT_pE194ori_cel_his. The plasmid was introduced into *E. coli* DH10B by electroporation. Ampicillin resistant transformants were screened by colony PCR. The plasmid was isolated by miniprep from positive clones and sequence was verified by DNA sequence analysis.

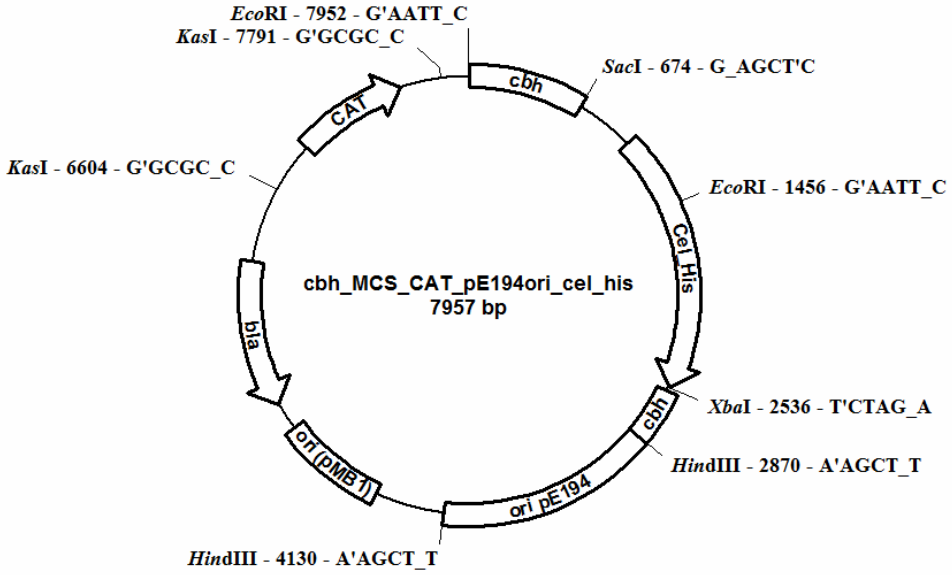


Figure 4.14: Map of the transfer vector cbh_MCS_CAT_pE194ori_cel_his.

The vector was transformed and integrated into the chromosome of *L. plantarum* by two step homologous recombination (see section 4.3.2). Culture supernatants and cell pellets were subjected to SDS-PAGE and Western blot analysis. The enzyme could neither be detected in the culture supernatants nor in the cell pellets. One explanation of the negative results could be the low expression level of the cellulase gene. As the PCR screening did not show clear positive results, it is possible that clones were not stable and got rid of the whole plasmid during the second step of recombination. Further experiments have to be done.

5 Conclusions

One aim of this work was the construction of a transfer vector permitting vector free cloning. It was possible to construct a transfer vector which contained an antibiotic resistance gene for selection in *L. plantarum* and a multiple cloning site (MCS) for easy replacement of different target genes flanked by regions, homologous to the chosen locus of integration. During the practical work, it turned out, that the *L. plantarum* wild type strain has an existing weak resistance to erythromycin. Therefore, a chloramphenicol resistance gene was used as a selection marker.

Another task was to establish an optimized transformation protocol for *L. plantarum*. Several protocols for the electrotransformation of different *Lactobacillus* strains have been published and it turned out that only selected strains could be transformed with high efficiency. For the *L. plantarum* strain the first transformation experiments were performed according to Aukrust and Blom (Aukrust & Blom 1992), leading to low transformation efficiency. After the optimization of the parameters of influence, including composition of the wash and electroporation solutions, the inclusion of cell wall weakening agents in the growth medium and electric conditions such as electrical pulse strength and length, a transformation efficiency of $3x10^2$ transformants/µg DNA could be achieved.

A further objective was the integration and expression of an endo-1,4-β-glucanase gene from *B. subtilis* in *L. plantarum*. The integration could be achieved by a two step homologous recombination, whereas the vector was integrated into the host chromosome by the first step of recombination and elimination of unwanted vector sequences by a second step of recombination. The resulting recombinant strain *L. plantarum* E-1,4-BG contains no more unwanted sequences like antibiotic resistance genes. The activity of the endo-1,4-β-glucanase which catalyzes the first step of cellulose degradation could be demonstrated by Congo red assay. To compare the acidification performance of the recombinant strain and the wild type strain, micro ensiling experiments are in progress.

6 References

Ahrné, S., Nobaek, S., Jeppsson, B., Adlerberth, I., Wold, A. & Molin, G. (1998): The normal Lactobacillus flora of healthy human rectal and oral mucosa. J Appl Microbiol **85,** 88-94.

Alegre, M., Rodríguez, M. & Mesas, J. (2004): Transformation of Lactobacillus plantarum by electroporation with in vitro modified plasmid DNA. FEMS Microbiol Lett **241,** 73-77.

Alvarez, M., Herrero, M. & Suárez, J. (1998): The site-specific recombination system of the Lactobacillus species bacteriophage A2 integrates in gram-positive and gram-negative bacteria. Virology **250,** 185-193.

Archibald, F. & Fridovich, I. (1981): Manganese and defenses against oxygen toxicity in Lactobacillus plantarum. J Bacteriol **145,** 442-451.

Aukrust, T. & Blom, H. (1992): Transformation of Lactobacillus strains used in meat and vegetable fermentations. Food Research International **25,** 253-261.

Axelsson, L., Lindstad, G. & Naterstad, K. (2003): Development of an inducible gene expression system for Lactobacillus sakei. Lett Appl Microbiol **37,** 115-120.

Boizet, B., Flickinger, J. & Chassy, B. (1988): Transfection of Lactobacillus bulgaricus protoplasts by bacteriophage DNA. Appl Environ Microbiol **54,** 3014-3018.

Béguin, P. & Aubert, J. (1994): The biological degradation of cellulose. FEMS Microbiol Rev **13,** 25-58.

de Ruyter, P., Kuipers, O. & de Vos, W. (1996): Controlled gene expression systems for Lactococcus lactis with the food-grade inducer nisin. Appl Environ Microbiol **62,** 3662-3667.

de Vos, W. (1999): Gene expression systems for lactic acid bacteria. Curr Opin Microbiol **2,** 289-295.

de Vos, W. & Vaughan, E. (1994): Genetics of lactose utilization in lactic acid bacteria. FEMS Microbiol Rev **15,** 217-237.

Derzelle, S., Hallet, B., Francis, K., Ferain, T., Delcour, J. & Hols, P. (2000): Changes in cspL, cspP, and cspC mRNA abundance as a function of cold shock and growth phase in Lactobacillus plantarum. J Bacteriol **182,** 5105-5113.

Dickely, F., Nilsson, D., Hansen, E. & Johansen, E. (1995): Isolation of Lactococcus lactis nonsense suppressors and construction of a food-grade cloning vector. Mol Microbiol **15,** 839-847.

Eijsink, V., Axelsson, L., Diep, D., Håvarstein, L., Holo, H. & Nes, I. (2002): Production of class II bacteriocins by lactic acid bacteria; an example of biological warfare and communication. Antonie Van Leeuwenhoek **81,** 639-654.

Gilkes, N., Henrissat, B., Kilburn, D., Miller, R.J. & Warren, R. (1991): Domains in microbial beta-1, 4-glycanases: sequence conservation, function, and enzyme families. Microbiol Rev **55,** 303-315.

Gosalbes, M., Esteban, C., Galán, J. & Pérez-Martínez, G. (2000): Integrative food-grade expression system based on the lactose regulon of Lactobacillus casei. Appl Environ Microbiol **66,** 4822-4828.

Götz, F., Elstner, E., Sedewitz, B. & Lengfelder, E. (1980a): Oxygen utilization by Lactobacillus plantarum. II. Superoxide and superoxide dismutation. Arch Microbiol **125,** 215-220.

Götz, F., Sedewitz, B. & Elstner, E. (1980b): Oxygen utilization by Lactobacillus plantarum. I. Oxygen consuming reactions. Arch Microbiol **125,** 209-214.

Helanto, M., Kiviharju, K., Leisola, M. & Nyyssölä, A. (2007): Metabolic engineering of Lactobacillus plantarum for production of L-ribulose. Appl Environ Microbiol **73,** 7083-7091.

Holo, H. & Nes, I. (1989): High-Frequency Transformation, by Electroporation, of Lactococcus lactis subsp. cremoris Grown with Glycine in Osmotically Stabilized Media. Appl Environ Microbiol **55,** 3119-3123.

Hols, P., Kleerebezem, M., Schanck, A., et al. (1999): Conversion of Lactococcus lactis from homolactic to homoalanine fermentation through metabolic engineering. Nat Biotechnol **17,** 588-592.

Johansen, E., Dickely, F., Nilsson, D. & Hansen, E. (1995): Nonsense suppression in Lactococcus lactis: construction of a "food-grade" cloning vector. Dev Biol Stand **85,** 531-534.

Joutsjoki, V., Luoma, S., Tamminen, M., Kilpi, M., Johansen, E. & Palva, A. (2002): Recombinant Lactococcus starters as a potential source of additional peptidolytic activity in cheese ripening. J Appl Microbiol **92,** 1159-1166.

Kandler, O. (1983): Carbohydrate metabolism in lactic acid bacteria. Antonie Van Leeuwenhoek **49,** 209-224.

Kim, Y., Han, K., Oh, S., You, S. & Kim, S. (2005): Optimization of technical conditions for the transformation of Lactobacillus acidophilus strains by electroporation. J Appl Microbiol **99,** 167-174.

Kleerebezem, M., Boekhorst, J., van Kranenburg, R., et al. (2003): Complete genome sequence of Lactobacillus plantarum WCFS1. Proc Natl Acad Sci U S A **100,** 1990-1995.

Kleerebezem, R. & Stams, A. (2000): Kinetics of syntrophic cultures: a theoretical treatise on butyrate fermentation. Biotechnol Bioeng **67,** 529-543.

Klyosov, A. (1990): Trends in biochemistry and enzymology of cellulose degradation. Biochemistry **29,** 10577-10585.

Kuipers, O., de Ruyter, P., Kleerebezem, M. & de Vos, W. (1997): Controlled overproduction of proteins by lactic acid bacteria. Trends Biotechnol **15,** 135-140.

Ladero, V., Ramos, A., Wiersma, A., et al. (2007): High-level production of the low-calorie sugar sorbitol by Lactobacillus plantarum through metabolic engineering. Appl Environ Microbiol **73,** 1864-1872.

Laemmli, U. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227,** 680-685.

Leenhouts, K., Buist, G., Bolhuis, A., et al. (1996): A general system for generating unlabelled gene replacements in bacterial chromosomes. Mol Gen Genet **253,** 217-224.

Leer, R., Christiaens, H., Verstraete, W., Peters, L., Posno, M. & Pouwels, P. (1993): Gene disruption in Lactobacillus plantarum strain 80 by site-specific recombination: isolation of a mutant strain deficient in conjugated bile salt hydrolase activity. Mol Gen Genet **239,** 269-272.

Llull, D. & Poquet, I. (2004): New expression system tightly controlled by zinc availability in Lactococcus lactis. Appl Environ Microbiol **70,** 5398-5406.

Madsen, S., Arnau, J., Vrang, A., Givskov, M. & Israelsen, H. (1999): Molecular characterization of the pH-inducible and growth phase-dependent promoter P170 of Lactococcus lactis. Mol Microbiol **32,** 75-87.

Makrides, S. (1996): Strategies for achieving high-level expression of genes in Escherichia coli. Microbiol Rev **60,** 512-538.

Nauta, A., van den Burg, B., Karsens, H., Venema, G. & Kok, J. (1997): Design of thermolabile bacteriophage repressor mutants by comparative molecular modeling. Nat Biotechnol **15,** 980-983.

O'Sullivan, D., Walker, S., West, S. & Klaenhammer, T. (1996): Development of an expression strategy using a lytic phage to trigger explosive plasmid amplification and gene expression. Biotechnology (N Y) **14,** 82-87.

Papagianni, M., Avramidis, N. & Filioussis, G. (2007): High efficiency electrotransformation of Lactococcus lactis spp. lactis cells pretreated with lithium acetate and dithiothreitol. BMC Biotechnol **7,** 15.

Pavan, S., Hols, P., Delcour, J., et al. (2000): Adaptation of the nisin-controlled expression system in Lactobacillus plantarum: a tool to study in vivo biological effects. Appl Environ Microbiol **66,** 4427-4432.

Sanders, J., Leenhouts, K., Burghoorn, J., Brands, J., Venema, G. & Kok, J. (1998): A chloride-inducible acid resistance mechanism in Lactococcus lactis and its regulation. Mol Microbiol **27,** 299-310.

Seegers, J. (2002): Lactobacilli as live vaccine delivery vectors: progress and prospects. Trends Biotechnol **20,** 508-515.

Serror, P., Sasaki, T., Ehrlich, S. & Maguin, E. (2002): Electrotransformation of Lactobacillus delbrueckii subsp. bulgaricus and L. delbrueckii subsp. lactis with various plasmids. Appl Environ Microbiol **68,** 46-52.

Simon, D., Rouault, A. & Chopin, M. (1986): High-efficiency transformation of Streptococcus lactis protoplasts by plasmid DNA. Appl Environ Microbiol **52,** 394-395.

Sixou, S., Eynard, N., Escoubas, J., Werner, E. & Teissié, J. (1991): Optimized conditions for electrotransformation of bacteria are related to the extent of electropermeabilization. Biochim Biophys Acta **1088,** 135-138.

Straume, D., Axelsson, L., Nes, I. & Diep, D. (2006): Improved expression and purification of the correctly folded response regulator PlnC from lactobacilli. J Microbiol Methods **67,** 193-201.

Sørensen, K., Larsen, R., Kibenich, A., Junge, M. & Johansen, E. (2000): A food-grade cloning system for industrial strains of Lactococcus lactis. Appl Environ Microbiol **66,** 1253-1258.

Sørvig, E., Mathiesen, G., Naterstad, K., Eijsink, V. & Axelsson, L. (2005): High-level, inducible gene expression in Lactobacillus sakei and Lactobacillus plantarum using versatile expression vectors. Microbiology **151,** 2439-2449.

Thompson, K. & Collins, M.A. (1996): Improvement in electroporation efficiency for Lactobacillus plantarum by the inclusion of high concentrations of glycine in the growth medium. Journal of Microbiological Methods **26,** 73-79.

Van Montfort, R., Slingsby, C. & Vierling, E. (2001): Structure and function of the small heat shock protein/alpha-crystallin family of molecular chaperones. Adv Protein Chem **59,** 105-156.

Venema, G. (1993): Dairy Foods: Molecular Biology and Genetic Modification of Lactococci. J Dairy Sci **76,** 2133-2144.

Vescovo, M. (1983): Conjugal Transfer of Broad-Host-Range Plasmid pAM31 into Enteric Species of Lactic Acid Bacteria. APPLIED AND ENVIRONMENTAL MICROBIOLOGY **46,** 753-755.

Wei, M.-Q. & Rush, C.M. (1995): An improved method for the transformation of Lactobacillus strains using electroporation. J Microbiol Methods **21,** 97-109.

Wells, J., Wilson, P. & Le Page, R. (1993): Improved cloning vectors and transformation procedure for Lactococcus lactis. J Appl Bacteriol **74,** 629-636.