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CD-Laboratory for Genetically Engineered Lactic Acid Bacteria

DEVELOPMENT OF GENETIC TOOLS FOR HETEROLOGOUS GENE EXPRESSION IN *L. PLANTARUM*

Supervisor

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„Ich bin immer noch verwirrt, aber auf einem höheren Niveau."

Enrico Fermi (1901 - 1954)
Nobel Prize in Physics

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Abstract

Lactic Acid Bacteria (LAB) are a heterogeneous group of Gram-positive bacteria with low G+C content. Lactobacillus is the largest genus, with over 150 species and can be divided into several groups based on genetic heterogeneity. Within the *Lactobacillus plantarum* group the diversity of phenotypical traits and preferred habitats still varies largely. *L. plantarum* CD033 was isolated from a stable silage in Austria. High transformation efficiencies (up to 10^9 cfu/µg DNA) make this strain highly attractive for strain improvement by genetic engineering e.g. for the expression of recombinant proteins.

This study deals with the development of genetic traits for *L. plantarum*. We have established a variety of suitable shuttle vectors, mainly based on high copy plasmids pCDLbu1 isolated from *L. buchneri* CD034. We tested endogenous constitutive promoters (e.g. P_{tuf33} and P_{tuf34}) in either high or low copy number vectors. The optimal spacer sequence between Shine-Dalgarno sequence and translation start of reporter gene mCherry was determined.

L. plantarum CD033 embodies a 7.9 kb plasmid pCD033, which was sequenced and annotated. Relative plasmid copy number of pCD033 was determined as well as the minimal stable replicon. Furthermore, we suggest replication by a novel theta mechanism. By providing a curing plasmid, and addition of sub-lethal doses of novobiocin, *L. plantarum* CD033 was cured of pCD033. A novel, plasmid free strain *L. plantarum* 3NSH was achieved, and evaluated for expression of recombinant proteins.

Inducible promoters further contribute to a comprehensive use of *L. plantarum* as production host. Therefore, we established and characterized three different promoter/repressor systems (namely P_{lacA}, P_{xylA} and $P_{laclSynth}$) regarding mCherry expression. We determined the preferred inducer (lactose, xylose and IPTG) and their required concentrations. Fluorescence signals and growth behavior were monitored with the BioLector® micro-fermentation system. The mCherry expression was inducible, but weak in comparison to a constitutive promoter and basal expression without inducer was observable. Furthermore, we successfully applied the T7 RNA polymerase for the first time in *L. plantarum* under control of an inducible promoter.

This study reveals a novel plasmid free *L. plantarum* strain and appropriate tools for biotechnological applications e.g. enhanced silage processes or recombinant protein production in a Generally Regarded as Safe (GRAS) microorganism.

Zusammenfassung

Milchsäurebakterien umfassen eine heterogene Gruppe Gram-positiver Bakterien mit geringem G+C Gehalt. Die größte Gattung (Lactobacillus) umfasst über 150 Spezies und kann anhand genetischer Varietät eingeteilt werden. Die *Lactobacillus plantarum* Gruppe ist phänotypisch sehr divers und das jeweils bevorzugte Habitat variiert stark. *L. plantarum* CD033 wurde aus einer stabilen Grassilage in Österreich isoliert. Eine hohe Transformationseffizienz (bis zu 10^9 kbe/µg DNS) macht *L. plantarum* CD033 attraktiv für Stammverbesserung und genetische Veränderungen z.B. für die Herstellung rekombinanter Proteine.

In dieser Thesis haben wir mehrere Shuttle-Vektoren, basierend auf dem *L. buchneri* CD034 Plasmid pCDLbu1, etabliert. Wir haben endogene konstitutive Promotoren (z.B. P_{tuf33} und P_{tuf34}) in Plasmiden mit hoher oder geringer Kopienzahl getestet. Die optimale Distanzsequenz zwischen Shine-Dalgarno und Translationsstart wurde ermittelt.

L. plantarum CD033 enthält ein 7,9 kb großes Plasmid pCD033, welches wir sequenzierten, charakterisierten und annotierten. Die Plasmidkopienzahl und das kleinstmöglich stabile Replikon wurden bestimmt und 8 codierende Sequenzen wurden gefunden. Anhand der erhaltenen Daten vermuten wir einen neuen theta-Replikationsmechanismus für dieses Plasmids. Mittels „curing plasmid“ und Zugabe subletaler Dosen eines „curing agents“ Novibiocin wurde *L. plantarum* CD033 von pCD033 befreit. Der Plasmid freie Stamm *L. plantarum* 3NSH wurde generiert und für die Expression rekombinanter Proteine evaluiert.

Induzierbare Promotoren erweitern die Verwendung von *L. plantarum*. Deshalb haben wir drei Promotor/Repressor Systeme (P_{lacA}, P_{xylA} and $P_{lacISynth}$) etabliert und in Bezug auf mCherry Expression getestet. Bevorzugte Induktoren (Laktose, Xylose und IPTG) und deren optimale Konzentrationen wurden mit dem BioLector® micro-fermentation System analysiert. Weiters konnten wir ein induzierbares System für die T7 RNA Polymerase etablieren und erstmals für *L. plantarum* beschreiben. Die etablierten Konstrukte sind induzierbar (gering verglichen mit einem konstitutiven Promoter) und basale Expression ohne Induktion war vorhanden.

Hiermit präsentieren wir einen neuen Plasmid-freien *L. plantarum* Stamm und Instrumente für dessen biotechnologische Verwendung z.B. für verbesserte Silage oder zur Produktion von rekombinanten Proteinen in einem als sicher eingestuftem GRAS („generally regarded as safe“) Mikroorganismus.

Contents

1 Scope of the study

L. plantarum is found in many ecological niches and used in a wide range of different applications (Douillard and de Vos, 2014; Malik et al., 2014; Mayo et al., 1989). Recently, *L. plantarum* was applied efficiently for surface display of antigens, which can be used for *in-vivo* vaccination and more (Fredriksen et al., 2012; Hols et al., 1997; Oh et al., 2007; Schultz et al., 2002). The economic potential of "generally regarded as safe" (GRAS) strains such as *L. plantarum* is manifold, and specific research is of great interest. Availability of effective as well as stable expression vectors is crucial.

Therefore, the scope of this study primarily comprises improvement and the development of genetic tools for this important strain *L. plantarum* CD033.

I) In the first part of this study, different constitutive promoters were tested and expression vectors were optimized for *L. plantarum* CD033 (Tauer et al., 2014).

II) In the second part, the single plasmid of *L. plantarum* CD033 (pCD033) was sequenced, characterized and annotated. This strain was cured from its plasmid yielding the plasmid free expression host *Lactobacillus plantarum* 3NSH. The plasmid pCD033 was sequenced and characterized (Heiss et al., 2015).

III) In the third part of the thesis different inducible promoter/repressor systems were tested for their functionality in *L. plantarum*. Based on the stable high copy number plasmid pCDLbu1 (Heinl et al., 2011) we analyzed mCherry (red fluorescent protein) under control of different promoter/repressor systems. Additionally, an inducible T7 based dual plasmid expression system was established for *L. plantarum* (Heiss et al., 2016).

Altogether, we optimized recombinant protein expression in *L. plantarum* and we expanded the toolbox of genetic engineering of a GRAS strain.

2 Introduction

2.1 Lactic Acid Bacteria

Lactic acid bacteria (LAB) are a heterogeneous group of Gram-positive bacteria with low G+C content. LAB include many families, such as *Leuconostocaceae*, *Streptococcaceae*, *Enterococcaceae*, *Carnobacteriaceae*, *Aerococcaceae* and *Lactobacillaceae* (Makarova et al., 2006). They are microaerophilic, non-sporulating acid-tolerant, and they ferment carbohydrates mostly to lactic acid. Apart from that, they are widely differing in biochemical, ecological, molecular-biological, and immunological properties.

LABs are commonly found in a variety of natural habitats, but they are also used to produce a variety of fermented food, enzymes and metabolites. Thereby safe, storable, organolepticically pleasing foodstuffs are generated such as fermented milk products (cheese, yogurt, and kefir), bread and cereals (sourdough and ogi), beverages (malolactic fermentation in wines), vegetables (sauerkraut, silage, kimchi) and preserved and ensiled fish products. Since their permanent use for mankind is as long as settlement itself, many LAB are 'generally regarded as safe` (GRAS). Lactic acid fermentation is one of the oldest techniques worldwide for the preservation of highly perishable raw materials. Rapid acidification was in ancient times probably the most important role of lactic acid bacteria since it inhibits outgrowth of pathogenic or putrefactive organisms, thus avoiding food spoilage. Lactic acid, the main product formed during sugar metabolism, is responsible for the characteristic fresh acid taste. It also contributes to a ´clean` fermentation process (Stiles and Holzapfel, 1997). The properties of LAB in fermented food mainly result from their carbohydrate metabolism, but the most interesting aspect is certainly the establishment of a unique characteristic flavor, texture, and wholesomeness as a result of their metabolic activities (Ardö, 2006; Freiding et al., 2012; Smit et al., 2005).

The preservation of food products by lactic acid bacteria is also exerted by the production of bacteriocins, antibacterial polypeptides acting on undesired pathogenic bacteria such as *Listeria* and *Clostridium* (Klaenhammer, 1988). Since fermented food is industrially manufactured on a large scale, so-called starter cultures (defined cultures containing strains with known properties) are now added to the fermenting material. The use of starter cultures has progressively replaced the traditional, often uncontrolled fermentation processes. As a direct

consequence, there is a growing interest in the development of new starter cultures and expression hosts.

LABs are mainly found in nutrient rich environments, such as milk, vegetables or the gastrointestinal tract (GIT) and mucosa of mammals. As a consequence, they have lost the ability to synthesize a lot of metabolites (Morishita et al., 1981). On the other side, they also produce many helpful and beneficial metabolites (Pessione, 2012). Some LAB strains have probiotic effects on human health, such as anti-cholesteremic effect by lowering the blood serum cholesterol level (de Roos and Katan, 2000; de Vries et al., 2005). They play important roles in improving lactose digestion, reducing gastrointestinal disorders and enhancing cellular immunity. The ability to adhere to specific regions of the GIT has prompted research aimed at the use of lactobacilli as vehicles for the delivery of biologically active compounds, such as immune-modulators, antibodies, enzymes and vaccines (Chen et al., 2014; Kajikawa et al., 2010; Mercenier et al., 2000; Wei et al., 2010). Otherwise, some lactic acid bacteria (e.g. *Streptococcus*) are also present in pathological conditions on mucosal surfaces of GI or genitourinary tracts. There are also reports of lactobacilli causing serious infections (Antony, 2000), and involvement in dental plaque formation and acidification of the oral cavity (Hamilton and Svensäter, 1998). However, beneficial effects of LAB predominate.

Gram-positive bacteria do not produce LPS (lipopolysaccharides) amongst other benefits, and are able to export proteins directly to the extracellular medium (García-Fruitós, 2012). In past years, research has focused on the development of new and promising alternative bacterial expression systems. Therefore, lactobacilli are desirable cell factories for the production of food-grade enzymes (Maischberger et al., 2010; Peterbauer et al., 2011). Subsequently, there is considerable interest in the development of genetic tools for efficient and controllable gene expression (Sørvig et al., 2005a).

At present, over 400 LAB plasmids have been isolated and studied. They fall into two functional categories: Some plasmids are cryptic and dispensable for host survival, and others encode enzymes involved in central cellular metabolisms of their hosts (conditionally essential to cell viability). LAB plasmids are extremely diverse in terms of size (0.87 to more than 250 kb), plasmid copy number (PCN) varies from 1 to more than 100 plasmids per cell, and they sometimes confer phenotypes to their hosts (Cui et al., 2015; Schroeter and Klaenhammer, 2009). The high transformation efficiencies of certain *Lactobacillus* strains allow for the successful transformation with ligation mixtures. The circumvention of intermediate cloning

hosts like *Escherichia coli* facilitates cloning procedures (Posno et al., 1991; Spath et al., 2012). The use of GRAS expression hosts together with food-grade expression systems and the use of enzymes derived from various probiotic (and therefore safe) strains is a forward-looking approach (Maischberger et al., 2010).

2.2 Lactobacillus plantarum

Lactobacillaceae are the largest family of the LAB, with over 150 species (number rising). They are heterogeneous and are grouped based on genetic relations, e.g. as the *L. salivarius*, *Pediococcus*, *L. plantarum*, *L. buchneri* group and many more (Salvetti et al., 2012). Figure 2 shows a dendrogram of LAB on the basis of concatenated alignment of ribosomal proteins and division into specific groups (Makarova et al., 2006). The genetic diversity within the *Lactobacillus plantarum* group is very high as well as their niches and phenotypic traits (Siezen and van Hylckama Vlieg, 2011). The occurrence of *L. plantarum* and its use ranges from vegetable, dairy and meat fermentations as well as silage. Moreover it is found in wines and ciders and the human GIT.

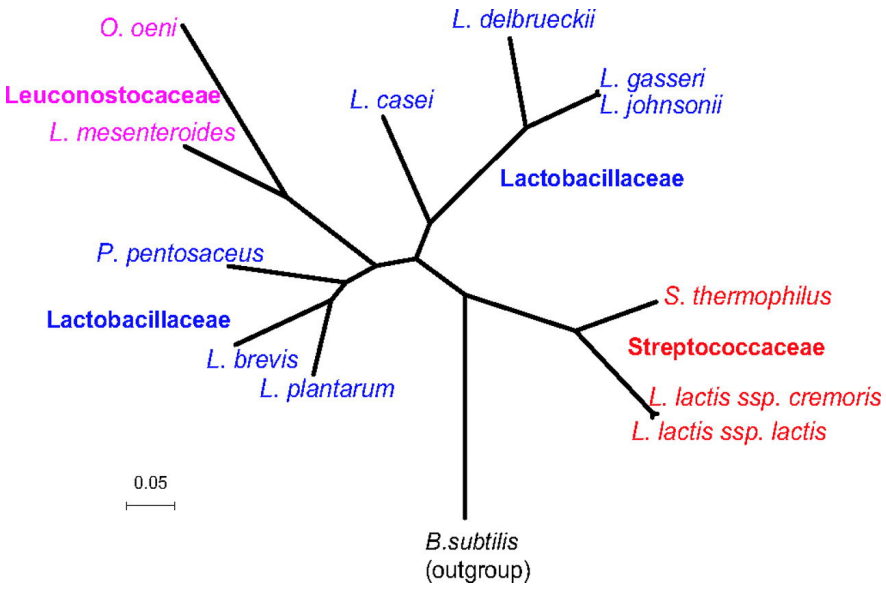


Figure 2: Dendrogram of Lactobacillales on the basis of concatenated alignment of ribosomal proteins and division into specific groups (Makarova et al., 2006).

L. plantarum WCSF1 (the type strain) has one of the largest genomes among LAB (3.3 Mb), a G+C content of 44.5 % and three plasmids of 1.9 kb, 2.3 kb and 36.1 kb (Kleerebezem et al., 2003). A summary of selected sequenced genomes of *L. plantarum* strains is shown in Table 1. *L. plantarum* is facultative hetero-fermentative, which means that hexoses can be fermented via the Embden-Meyerhof-Parnas (EMP) pathway (glycolysis) or the phosphoketolase pathway, leading to homolactic and heterolactic fermentation profiles (Kandler, 1983). No complete citric acid cycle is encoded even though several enzymes of this pathway are present. Apart from that, a plethora of genes for sugar uptake and – transport are encoded (25 complete PTS sugar transport systems), as well as more than 200 putative extracellular proteins (Figure 3). This contributes to the large flexibility in interactions with the environment. Generally, genes are predominantly transcribed in the direction of replication, which is a feature observed in many genomes of Gram-positive bacteria (Kleerebezem et al., 2003).

Table 1: Summary of sequenced genomes of *Lactobacillus plantarum* strains (Siezen and van Hylckama Vlieg, 2011)

	WCFS1	**JDM1**	**ST-III**	**ATCC 14917**	**NC8**	**KCA1**
Genome size (bp)	~3.3 Mb	~3.2 Mb	~3.2 Mb	~3.2 Mb	~3.2 Mb	~3.4 Mb
contigs	1	1	1	36	67	84
GC%	44.5	44.7	44.6	44.0	45.0	45.3
CDS	3007	2948	2996	3154	~3000	~3200
plasmids	3	2	1	?	?	0
Accession code	NC_004567	CP001617	NC_014554	NZ_ACGZ02000000		
source	human saliva	grass silage?	kimchi	pickled cabbage	grass silage	human vagina
reference	(Kleerebezem et al., 2003)	(Zhang et al., 2009)	(Wang et al., 2011)	Human Genome Sequencing Center, Baylor College of Medicine, Houston, Texas, USA	Axelsson et al, unpublished	Anukam et al, unpublished

Strains with probiotic effects on mice have been reported (Schultz et al., 2002). Some strains are also marketed as probiotics and claim to provide health benefit for humans (Alander et al., 1999; Cunningham-Rundles et al., 2000; Johansson et al., 1998). Different *L. plantarum* strains are known to pass the stomach and GIT, showing a high tolerance to hydrochloric acid and bile salts (de Vries et al., 2005). *L. plantarum* is also frequently found as one of the dominating *Lactobacillus* species in the small intestine of man (Ahrné et al., 1998). Furthermore, *L. plantarum* strains can adhere to human cells in a mannose-related manner. This supports designing strains for delivering desirable and potentially health-promoting proteins to the GIT (Vesa et al., 2000). Both, the potential probiotic effects of *L. plantarum* and the high survival rate during the passage of the GIT (Vesa et al., 2000) support for *in-situ* delivery of therapeutically interesting proteins (Fredriksen et al., 2012; Mathiesen et al., 2009).

Many species of *Lactobacillus* contain plasmids which vary widely in size and number, and gene content (Siezen and van Hylckama Vlieg, 2011; Wang and Lee, 1997). *L. plantarum* contains the largest plasmids in the genus *Lactobacillus* (Guidone et al., 2013). At present, at least 56 plasmids form *L. plantarum* have been sequenced and encode some important properties, including antibiotic resistance, exopolysaccharide biosynthesis, chloride or potassium transport, bacteriophage resistance, as well as bacteriocin production (Cui et al., 2015; Heiss et al., 2015). *L. plantarum* LPC25 was found to contain 16 plasmids (Ruiz-Barba et al., 1991). Some plasmids are between 1.2 kb and 169 kb from *L. plantarum* LL21 and LL2 respectively (Mayo et al., 1989). Many small plasmids from *L. plantarum* replicate via the rolling-circle replication (RCR) mechanism, while some large plasmids are shown to replicate via the theta mechanism (Chen et al., 2014; Feld et al., 2009). It is suggested that plasmid recombination among *Lactobacillus* plasmids occurs via horizontal gene transfer. Hence, some of these plasmids could also have evolved from common ancestors (Bringel et al., 1989; Wang and Lee, 1997).

In *L. plantarum* and some other LAB, high amounts of manganese up to 30 mM accumulate intracellularly due to compensate for the absence of superoxide dismutase (SOD) in these species (Archibald and Fridovich, 1981). Therefore, *L. plantarum* requires high concentrations of manganese in the standard MRS medium to be able to grow successfully (Hantke, 2005; Kleerebezem et al., 2003). Under certain circumstances, including glucose limitation conditions, *L. plantarum* is able to utilize oxygen. Growth stagnation under aerobic condition was described by Stevens and colleagues (Stevens et al., 2008). The high degree of fumarate reductase redundancy suggests that *L. plantarum* harbors a rudimentary electron transport chain. The

metabolic model for *L. plantarum* WCSF1 predicts that bicarbonate is used for the production of purines and pyrimidines (Teusink et al., 2005).

L. plantarum has already been used extensively for recombinant protein production, ranging from β-galactosidase (Halbmayr et al., 2008) to phytase (Kerovuo and Tynkkynen, 2000). Additionally, production of metabolites such as sorbitol (Ladero et al., 2007) and silage relevant enzymes, such as endo-1,4-beta-glucanase in *L. plantarum* B41AC1 have been reported (Rossi et al., 2001). Different *L. plantarum* strains have different secretion capacities (Hols et al., 1997).

2.2.1 Sugar metabolism in *L. plantarum*

The metabolic routes by which sugars are utilized is the classical EMP for the homofermentative LAB and the oxidative pentose phosphate pathway for the obligate or facultative heterofermentative LAB (Kandler, 1983). All carbohydrates metabolized by the heterolactic pathway (also called the phosphoketolase pathway or short PKP) are first converted by appropriate enzymes into xylulose-5-phospate that is then fermented into equimolar amounts of lactic acid and acetic acid. Among LAB, usually the *lactobacilli* have a greater capacity to ferment a broader range of carbohydrates.

During growth on glucose *L. plantarum* almost shows a homolactic fermentation and glucose is degraded via the EMP pathway leading to pyruvate, which is subsequently converted to approximately equimolar amounts of D- and L-lactate by the activities of stereospecific lactate dehydrogenase (Ldh) enzymes (Ferain et al., 1994). In addition to fermentation of carbohydrates, production of metabolites e.g. formate, acetate, ethanol, acetoin and 2,3-butanediol is ongoing (Kleerebezem et al., 2002). A high number of genes encode putative sugar transporters in *L. plantarum* WCSF1 (Figure 3, black color). Interestingly, many sugar transport and metabolism related genes are clustered near the origin of replication. The majority of these transporters are predicted PEP (phosphoenolpyruvate)-dependent sugar phosphor transferase systems (PTSs). In many cases these PTS genes are genetically linked to genes of enzymes involved in degradation specifically of the transported sugar. In addition to PTSs, the *L. plantarum* WCSF1 genome encodes 30 transporter systems predicted to be involved in the transport of carbon sources.

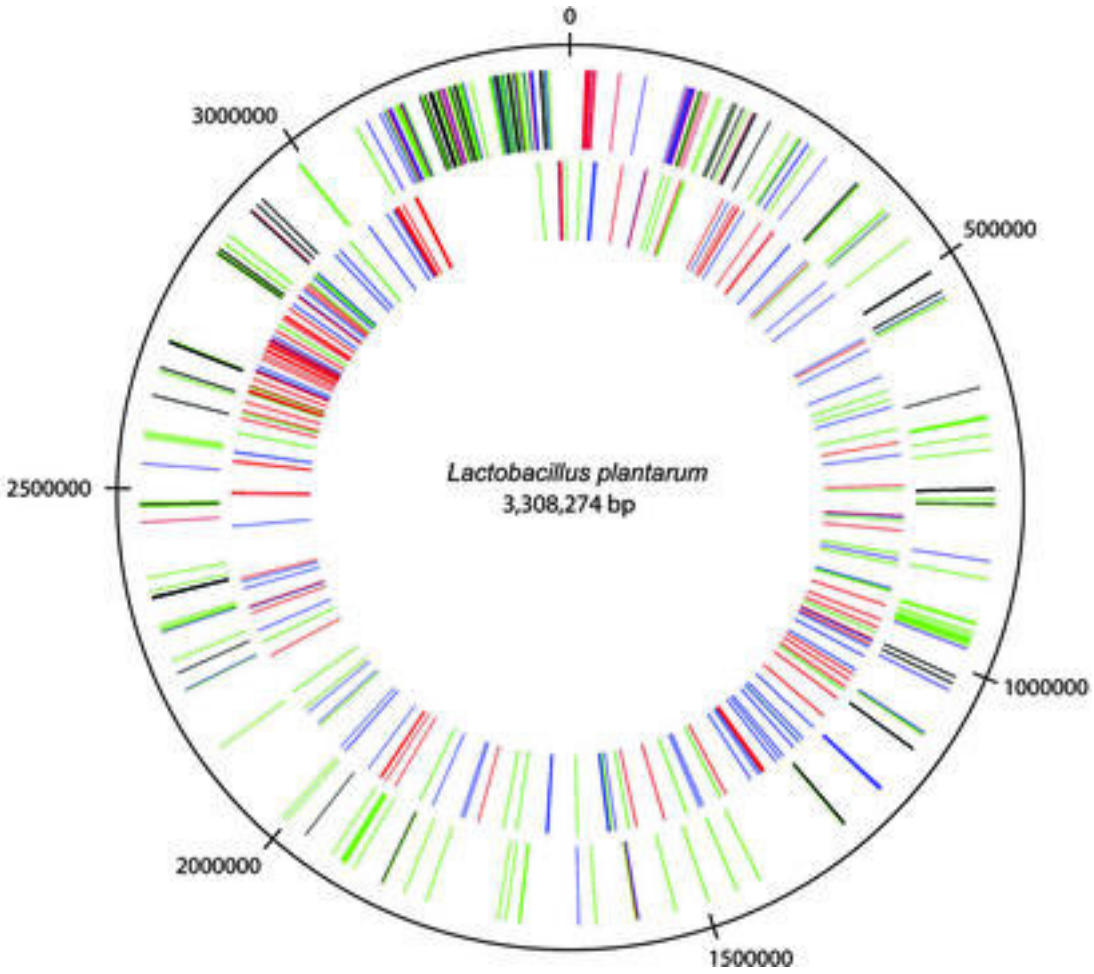


Figure 3: Nonrandom distribution of genes belonging to specific functional categories in the *L. plantarum* chromosome. The outer circle contains all genes encoding proteins involved in sugar transport (PTS are colored black, other transporters are colored blue), sugar metabolism (green), and biosynthesis and/or degradation of polysaccharides (red). The inner circle contains all genes predicted to encode secreted proteins. Red: signal peptides; green: N-terminal lipoprotein anchor; blue: N-terminal signal anchor sequence (Kleerebezem et al., 2003).

2.2.2 Utilization of lactose

L. plantarum uses lactose as main carbon source, which can be converted to lactic acid. Its frequent occurrence in dairy products supports the high abundancy of sugar related genes (Kleerebezem et al., 2003; Siezen et al., 2012). Therefore it is reasonable that *L. plantarum* WCSF1 encodes two ß-galactosidases. The *lacL* and *lacM* genes encode the small and large subunit of a β-galactosidase, which is potentially not regulated. The *lacA* gene encode the β-galatosidase-I which is (likewise to the *lacS* lactose permease gene) regulated by the *lacR* encoded transcription regulator of the LacI family.

Experiments carried out with the *L. plantarum* promoter region showed that the *lacLM* genes are expressed in glucose and lactose, with a 6-fold increase of activity in the presence of lactose

compared to that found for glucose (Mayo et al., 1994). In *Leuconostoc lactis lacL* and *lacM* are translationally coupled (David et al., 1992) and constitutively expressed (Vaughan et al., 1996). Also in *L. delbrueckii* subsp. *bulgaricus* ATCC 11842^{T} the β-galactosidase expression is constitutive (Leong-Morgenthaler et al., 1991). The *lac* operon is also subject to catabolite repression by CcpA in for example *L. casei* (Monedero et al., 1997). Inactivation of CcpA resulted in de-repression of transcription initiation of the *lac* operon.

The transcriptional regulator of the *lac* operon (LacR) is positioned upstream of *lacA*, organized in a divergent orientation such that the two promoters are in a back-to-back configuration. LacR as transcription factor is both responsible for repression of the *lac* operon and activating its own transcription in *L. lactis* (van Rooijen and de Vos, 1990; van Rooijen et al., 1992). During growth on glucose (or non-induced state) the binding of LacR to the operator (located in the *lac* operon promoter) represses transcription of the *lac* promoter, but activates transcription of *lacR*. Binding of the inducer (e.g. lactose) to LacR results in dissociation of the LacR operator complex and expression of the *lac* operon may proceed (van Rooijen et al., 1993).

2.2.3 Carbon catabolite repression

Carbon catabolite repression (CCR) in bacteria is a regulatory mechanism whereby a rapidly metabolizable carbon source, often glucose, in the growth medium inhibits utilization of other substrates (Gunnewijk et al., 2001; Stülke and Hillen, 1999). Transcription factors (TFs) mediate preferences in the utilization of certain carbohydrates over others. The prioritization involves both repression (and activation) of catabolic genes in the absence (or presence) of a related or favored substrate.

CCR is regulated via CcpA (catabolite control protein A), which is a member of the LacI-GalR family of TF. CcpA is the only LacI-family member present in all sequenced *Firmicutes* genomes. It typically exerts its action via a catabolite responsive element (*cre*) which is positioned near the promoter region of specific genes and either represses or activates their transcription (Hueck et al., 1994; Weickert and Chambliss, 1990). CcpA also functions as a transcriptional regulator involved in the global metabolic control. Another critical factor in catabolite repression is HPr (histidine-containing phosphocarrier protein), which has additional functions besides acting as an intermediate in the phosphate-transfer chain of the sugar PEP-PTS. The *ccpA* gene has been characterized in *L. plantarum*. Inactivation of *ccpA* resulted in relief of the

negative effect mediated by glucose on the activity of several catabolic enzymes (Chaillou et al., 1998; Francke et al., 2008; Muscariello et al., 2001). The degeneracy of the CcpA operator motif indicates it should act at relatively higher concentrations with respect to LacI and other relatives. Therefore, CcpA seems to act as activator as well as repressor depending on the actual promoter, emphasizing the 'global' role of CcpA (Chaillou et al., 2001). *Cre* sites have been detected in the promoter regions of many genes, such as *amyA* α-amylase and *bglH* phosphor-β-glucosidase in *L. plantarum* (Giraud and Cuny, 1997; Marasco et al., 1998).

11 out of 12 LacI-family TFs were found to be associated with active carbohydrate transport systems (Francke et al., 2008). An unexpected finding has been their transcriptional activation of genes and operons involved in glycolysis and lactic acid production (Luesink et al., 1998; van den Bogaard et al., 2000). Glucose regulated inhibition of induction of the enzymes required for lactose and galactose utilization is exerted at the level of inducer transport and therefore the mechanism of glucose effect is through catabolite inhibition rather than catabolite repression (Hasan and Durr, 1974). For example, D-glucose inhibited induction of β-galactosidase in *L. plantarum*, whereas D-galactose or thiomethyl galactoside (TMG), lactose (to a much lesser extent), isopropyl thiomethyl galactose (IPTG) and D-fucose induced enzyme expression. Glucose exerts its effect through its ability to exclude entry of galactose or lactose into the cell. Neither galactose nor lactose severely affected the uptake of glucose by pre-induced cells.

2.3 LAB Plasmids: replication, stability and vector applications

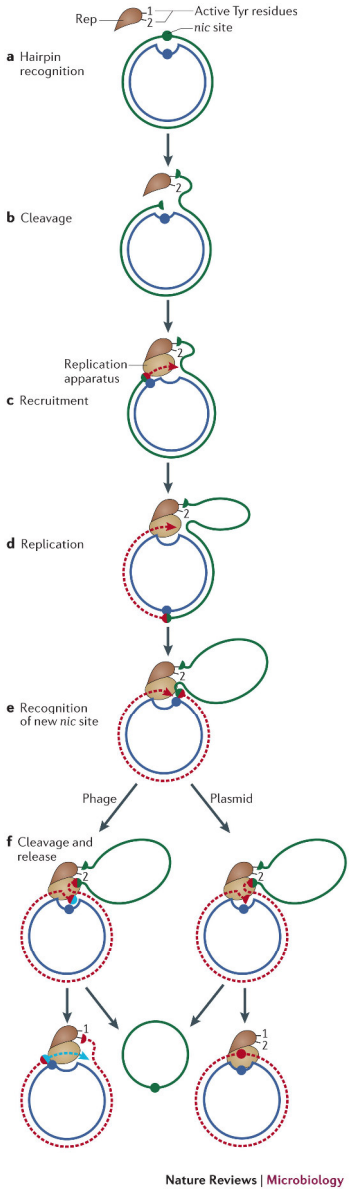
The availability of plasmid DNA sequence data and information on the minimal replicons has provided valuable insight for the use of LAB plasmids for the construction of LAB expression vectors as well as for genetic engineering. The mode of replication has an important impact on some characteristics of plasmid-derived vectors, namely host range, stability, and copy number (Shareck et al., 2004). Among three replication modes known for bacterial plasmids, most lactobacilli plasmids duplicate either via a sigma (rolling-circle) or via a theta mechanism (Khan, 2005). The third replication mechanism is based on strand displacement, but is not that frequently found in LAB plasmids. Plasmids from the rolling circle replication (RCR) and of the theta replication classes have been used to construct plasmid vectors (Aleshin et al., 1999; Leer et al., 1992).

2.3.1 Plasmid replication by the rolling circle mechanism

RCR depends on the involvement of three elements: a double strand origin (*dso*; originally termed as positive origin of replication), a replication initiator protein (Rep), and a single strand origin (*sso*; minus origin). Replication proceeds via two distinct phases: first, a single-stranded copy is made and second this is converted into double-stranded DNA (leading and lagging strand replication). The Rep protein acts as an endonuclease and topoisomerase, it recognizes the *dso* origin sequence and introduces a nick to initiate replication. The plus DNA strand is displaced and a new plus strand is synthesized by 3´-OH extension from the nick. This process requires host cell functions that include DNA III, ssDNA-binding protein, and a helicase. The DNA fragment containing plus origin and *rep* is called the replicon. An overview is given in Figure 4.

Rep recognizes a termination sequence that overlaps the origin sequence, producing a second nick to generate one fully replicated strand together with a single-stranded monomer of the displaced strand. The ends of the single-stranded DNA (ssDNA) are ligated to form a circle and conversion to double-stranded DNA (dsDNA) is mediated. This involves host factors that recognize a distinct *sso* where dsDNA synthesis starts. Characteristically, the plus origin is located upstream or within *rep*. Both the minus origin and the Rep protein exhibit sequence conservation across a large family of plasmids from diverse bacterial sources. Minus origins contain imperfect inverted repeats to form palindromic structures (del Solar et al., 1998; Gruss and Ehrlich, 1989).

Different types of RCR plasmids can coexist in the same species. Within the same group of plasmids, Rep proteins showed an extensive homology and are trans-acting (del Solar et al., 1998; Gruss and Ehrlich, 1989; Wang and Lee, 1997). The availability of Rep is rate limiting and this is the target used by the cell to control plasmid replication and copy number. Different mechanisms of plasmid replication and regulation are possible and are reviewed by del Solar and colleagues (1998).



RCR plasmids generally have a smaller replicon size (10 kb) due to the single strand intermediates. Therefore, cloning vectors based on RCR plasmids are preferably used for insertion of rather small DNA fragments. Plasmid instability is likely to increase with insert size (Kiewiet et al., 1993). The distribution of RCR plasmid molecules at cell division appears to be random (Novick, 1987). They are more prone to problems of both segregational and structural instability (compared to theta replicating plasmids), resulting in more frequent plasmid loss over successive generations and the deletion (or rearrangement) of specific sequences. This also contributes to and enhances the generation of high molecular weight multimers. Segregational instability of RCR plasmids has been attributed to the inefficient conversion of single-stranded DNA molecules to double-stranded molecules in second strand synthesis (Leer et al., 1992; Shimizu-Kadota et al., 1991), or to infidelity at various stages of the replicative process (Gruss and Ehrlich, 1989; Pouwels and Leer, 1993). In contrast, they have generally higher PCN and wider host ranges than theta replicating plasmids.

Figure 4: Schematic overview of plasmid rolling circle replication (Chandler et al., 2013).

2.3.2 Plasmid Replication by the Theta mechanism

In contrast to RCR plasmids, theta-type plasmids replicate by means of a double-stranded rather than a single-stranded replication intermediate, which results in better structural and segregational stability. Furthermore, they can accommodate large inserts (Kiewiet et al., 1993). A huge number of theta-replicating plasmids have been isolated and characterized (Shareck et al., 2004) and categorized into six classes of theta replicons termed Class A to F (Cui et al., 2015). Some families are restricted to specific species, but in general DNA synthesis of theta plasmids is bidirectional, may initiate from multiple origins and produces a double-stranded DNA replication intermediate. Single-stranded DNA is not formed; the plasmid molecule retains a circular conformation, DNA synthesis is primed at closely located sites within the origin of

replication, resulting in high segregational stability. Features which are typical for theta-replicating plasmids include an AT-rich region with multiple direct and inverted repeats, and several DnaA boxes (binding sites for host DnaA initiator proteins) were found (del Solar et al., 1998).

Additionally, species-specific theta plasmids with a narrow host-range are of interest for the construction of food-grade vectors. Theta-type plasmids provide a better backbone for vectors for gene expression and are suitable for protein vaccine expression in *lactobacilli* (Chen et al., 2014). Potential drawbacks are low PCN and therefore low gene dosage resulting in lower expression levels.

2.3.3 Plasmid vectors and plasmid incompatibility

The successful introduction of heterologous DNA into any species of LAB is dependent amongst others on the host strain and plasmid vector employed. As a result, once novel LAB strains are isolated, they should be investigated for harboring compatible plasmids in order to develop DNA cloning and gene expression tools for studying their biology and biotechnological applications. Certain strains of LAB appear to be recalcitrant to all methods of gene transfer in the laboratory. For a proportion of bacteria, the failure to recover recombinants might be attributable to problems of plasmid incompatibility or to the presence of an active restriction-modification system in the host bacterium (Novick, 1987; Posno et al., 1991).

Plasmids harboring identical or similar replicons will compete with each other during replication and partitioning of daughter plasmids in cell division. Such plasmids are incompatible; while one is usually maintained, the other is segregationally unstable and ultimately lost from the bacterial population. Thereby, only specific heterologous plasmids (and phenotypic traits) are retained for a cell. An attempt to avoid problems of incompatibility is to use strains cured of their indigenous plasmids. Loss of a native plasmid might also have adverse effect on the properties of industrial strains of LAB.

2.3.4 Plasmid addiction systems

Plasmid addiction systems (PAS) prevent the survival of cells that lost their plasmids (Zielenkiewicz and Cegłowski, 2001). Such systems were described for Gram-positive as well as Gram-negative bacteria encoded mainly on the plasmid, but can also be found on the chromosome (Siezen et al., 2012). Three major groups of PAS are described (Figure 5) according to their principle of function: (i) toxin/antitoxin (TA)-based system, (ii) metabolism-based system (who can be divided into catabolism- and anabolism-based system) and (iii) operator repressor titration (ORT) system. Some entirely synthetic systems are already established (Kroll et al., 2010). It was reported for TA systems that they are highly abundant in free-living bacteria (Pandey and Gerdes, 2005).

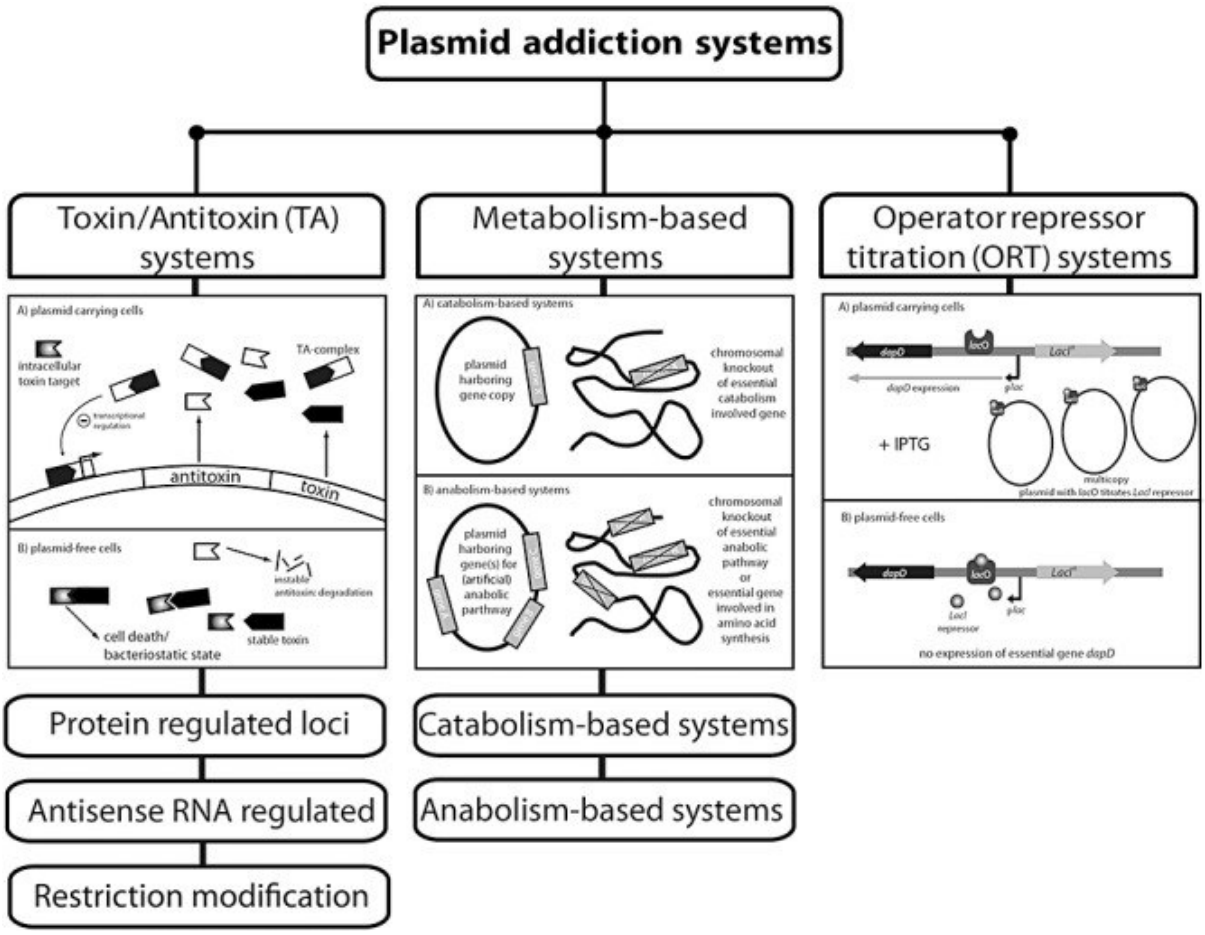


Figure 5: Overview and classification of the most common plasmid addiction systems (PAS) depending on their functionality and principle of mechanism. Toxin/antitoxin (TA) systems contain three separate subgroups with protein regulated loci, antisense RNA-regulated mechanism or specific restriction modification. The general function of this group is illustrated above by the mechanism of TA systems. Metabolism-based systems are divided into catabolism and anabolism-based systems depending on their target pathway and complementation principle. The operator repressor titration (ORT) system is emphasized as a stand-alone group (Kroll et al., 2010).

The principle of the TA based system is the counteraction of two proteins from which one is a stable toxin and the other is an unstable antitoxin. Three different groups can be distinguished: antisense RNA-regulated loci, protein-regulated loci and restriction modification systems (Fukuda et al., 2008; Gerdes et al., 1997; Handa et al., 2000; Jensen and Gerdes, 1995). In the case of plasmid loss, the unstable antidote is degraded rapidly, whereas the durable toxin is still active, leading to cell death. Thus TA systems are favoring stable plasmid maintenance and are assumed as active support for stable maintenance of low copy-number plasmids (Dmowski and Jagura-Burdzy, 2013; Sørvig et al., 2005b). Additionally, they are involved in cell stress conditions or biofilm formation (Kroll et al., 2010).

There is a strong need for unconventional antibiotic-free systems which still maintain plasmid stability. TA systems have been used e.g. for implementing the production of compounds based on enhanced plasmid stability (Kroll et al., 2010). Additionally, they were used as positive selection marker instead of antibiotics (Hägg et al., 2004). Therefore, TA systems are novel alternatives for pharmaceutical production processes where the use of antibiotics is limited.

2.3.5 Cloning Vectors

Plasmid vectors must be able to replicate in their host cells, except those intended for chromosomal integration. Autonomously replicating plasmids are often preferred, compared to genomic integration, because many copies of the target gene support for the desired phenotype. LAB vectors carry a Gram-positive replicon, or are derived from endogenous plasmids. In general it is easier to clone DNA in *E. coli* than in species of LAB. Consequently, bi-functional shuttle vectors containing two origins of replication (one for *E. coli* and one for LAB) and at least two selectable markers are used for cloning in E. coli and transformation of the final plasmid into LAB (de Vos, 1999; Pouwels and Leer, 1993; Wada et al., 2009; Wang and Lee, 1997). For *L. plantarum* CD033 it was shown that a direct cloning approach is possible, thus circumventing *E. coli* as intermediate host (Spath et al., 2012).

2.4 Heterologous gene expression in Lactic Acid Bacteria

The usage of LAB for recombinant gene expression and as novel expression hosts is, due to their specific properties, steadily rising. The cloning of heterologous genes with their native promoter and Shine-Dalgarno sequences in LAB often results in low levels of recombinant

protein. Additionally, translation efficiency may be influenced by inaccurate codon usage. This correlation is based on the circumstance that genes with high expression levels have strongly biased usage of synonymous codons (Mathiesen et al., 2004). Comparison of various published results is not straightforward because of differences in the genetic set-up of the expression systems, and because of variations between different *Lactobacillus* strains (e.g. efficiency of transcription and translation signals) (Pavan et al., 2000; Pouwels and Leer, 1993).

Whichever plasmid/promoter system is used, plasmid based high level protein expression causes a metabolic burden for the host cell. Energy and biomass precursors such as amino acids and nucleotides are directed away from the host cell metabolism (Carneiro et al., 2013). Additionally, the plasmid copy number augments during the cultivation process as the plasmid replication mechanism is influenced by the recombinant protein expression. This further increases the metabolic load on the bacterial host cell and triggers the stress response system. Every recombinant protein interacts with the host cell metabolism individually.

2.4.1 Gene Transcription and Promoter Organization

The transcription of a gene is initiated by RNA polymerase, which binds to the promoter. The σ-factor recognizes and binds to conserved hexamers located at positions -35 (TTGACA) and -10 (TATAAT) with respect to the transcription initiation site. These promoter hexamers are frequently separated by a spacer of 17 ± 1 nucleotides (typically rich in A and T residues), and often harbor a TG motif upstream of the -10 hexamer (Garmyn et al., 1995; Labarre et al., 1996; McCracken and Timms, 1999; Quirasco et al., 1999). A schematic overview of a Gram-positive transcription start is pictured in Figure 1.

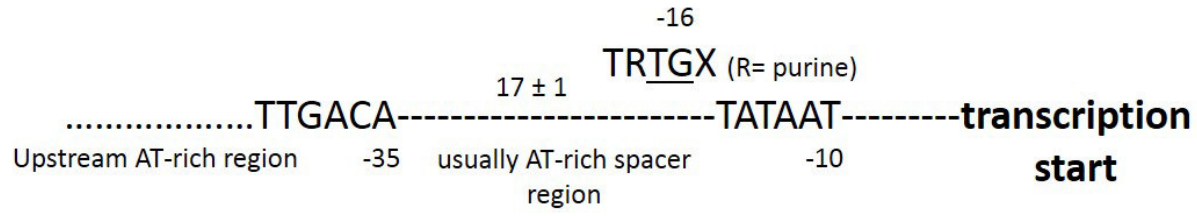


Figure 1: Schematic organization of a Gram-positive promoter

It is likely that promoter efficiency depends on the interaction of RNA polymerase with multiple sequences within the promoter region. The effect of the sequences is dependent on both the strain of *Lactobacillus* and on the specific sequence of the two consensus regions. (Platteeuw et

al., 1994). Similarly it was shown that the AT-rich region upstream of the -35 sequence of the *lac* promoter has been shown to enhance the efficiency of transcription initiation in *L. lactis* (van Rooijen et al., 1992).

The presence of a TG motif (-16 region) is of considerable importance in Gram-positive organisms (Figure 1, underlined), but has also been found in a limited number of *E. coli* promoters (Voskuil and Chambliss, 1998). Hence, the conservation frequency is varying between species and is strain specific (McCracken and Timms, 1999). This TG motif is not essential for promoter activity, but might enhance a rate limiting reaction, for example, docking of RNA polymerase near the -35 region. It is proposed that the TG motif is only of benefit in the context of weak -35 and -10 hexamers (in *B. subtilis*). The presence of both a consensus -35 hexamer and a TG motif may overstabilize transcription intermediates (McCracken and Timms, 1999; Voskuil and Chambliss, 1998).

Contrarily, any increase in promoter strength might be disrupted of later stages of transcription initiation (e.g. promoter clearance) (Garmyn et al., 1995; Labarre et al., 1996; Quirasco et al., 1999). This highlights the importance of using dedicated strain specific promoters for optimizing the expression of heterologous genes in different species of LAB (Platteeuw et al., 1994).

2.4.2 Translation of mRNA

The translation of mRNA in prokaryotes is initiated by the binding of the small subunit of a ribosome to the ribosome-binding sequence (RBS) or Shine-Dalgarno sequence. The RBSs of Gram-positive bacteria are often more extensive than those of *E. coli* (AGGA) (Moran, 1992). Analysis of sequences has shown that the majority of putative Shine-Dalgarno sequences in *Lactobacillus* species contain the highly conserved sequence AGGAGG (Pouwels and Leer, 1993) with variable length and extent of complementarities to the 16S rRNA sequence (3´-CUUCUU-5´). An introduction of a ´5´-box` upstream of the Shine-Dalgarno sequence improved antigen production by a factor of 10 in *L. plantarum* (Hols et al., 1997). Variations of spacer sequence between RBS and start codon has obvious influence on heterologous gene expression in *L. plantarum* (Tauer et al., 2014).

2.4.3 Inducible promoters

For many applications, the ability to control the production of recombinant proteins in LAB will be desirable. In bacteria, genes are regulated at both, the level of transcription and at the level of translation through inducer and repressor systems, anti-terminators, RNA binding proteins, and binding of anti-sense RNA to mRNA species. A number of expression systems responsive to environmental stimuli, inducers, or repressors have been described (de Vos et al., 1997; Kuipers et al., 1997).

Several inducible expression systems used in LAB have been described and reviewed (mostly in *L. lactis*). Controlled gene expression systems can be based on promoters controlled by sugars (e.g. *lac* promoter) (MacCormick et al., 1995; Simons et al., 1990), salt (*gadC*) (Sanders et al., 1998), temperature up-shift (*tec* phage promoter) (Nauta et al., 1996), pH decrease (P_{170} promoter) (Madsen et al., 1999), or phage infection (phi31 promoter) (Walker and Klaenhammer, 1998).

To fulfill food-grade criteria, inducing factors should be acceptable in foods, such as small inorganic molecules (salts), organic molecules (e.g. sugars or fatty acids), or proteinaceous compounds originating from LAB. Moreover changes in growth conditions including pH, temperature, aeration, or even phage infection would be an acceptable way to induce gene expression.

A well-established and efficient system for inducible expression for LAB is the NICE (nisin controlled expression)-system, based on the bacteriocin nisin (Kuipers et al., 1995; Shareck et al., 2004). An alternative expression system, the so-called pSIP system (Sørvig et al., 2003), was constructed for *Lactobacillus* spp. based on the promoter and regulatory genes involved in the production of the bacteriocins sakacin-A (Axelsson and Holck, 1995) and sakacin-P (Hühne et al., 1996). Expression of the GOI (gene of interest) is induced by the addition of a peptide pheromone. The applicability of the sakacin-based expression systems was shown for the over-production of enzymes such as β-glucuronidase and aminopeptidase in several *Lactobacillus* hosts, giving typically high induction results (Halbmayr et al., 2008; Sørvig et al., 2005a).

2.4.4 Constitutive promoters

Constitutive promoters have been studied with chloramphenicol acetyltransferase (CAT) (van der Vossen et al., 1985), sugar hydrolases (Simons et al., 1990), green fluorescent protein (only

for strong promoters) (Geoffroy et al., 2000), mCherry red fluorescent protein (Tauer et al., 2014) and luciferase (*luxAB*) genes (for cell extracts only) (Corthier et al., 1998). Constitutive expression of an *alr* (alanine racemase) gene has been studied in a *L. plantarum alr* deletion mutant (Bron et al., 2004). Moreover, a synthetic promoter library was established and analyzed in *L. plantarum* (Rud et al., 2006).

2.4.5 T7 RNA polymerase for orthologous gene expression

The most frequently used plasmid based expression systems in *E. coli* is the pET vector system (Novagen, EMD Millipore). This vector system comprises over 42 vector types which have different antibiotic resistance markers, different multiple cloning sites (MCS) and a variety of fusion tags which can be fused to the protein of interest. However, they all share the same pBR322 origin of replication and the GOI is always expressed under control of a T7 promoter and transcribed by a T7 RNA polymerase. This T7-phage originating RNA polymerase transcribes fivefold faster than the native *E. coli* polymerase and is integrated onto the host cell genome of thereafter so called DE3 strains, leaving the T7 RNA polymerase under control of a lacUV5 promoter. As soon as the lacUV5 promoter is induced with the lactose analogue IPTG, T7 RNA polymerase is expressed and starts to transcribe the GOI, which is on another plasmid under control of the T7 promoter and *lac* operator. In combination with *lacI* repressor gene, this sequential mode of action enables a tight control of the recombinant protein expression (Rosenberg et al., 1987; Studier et al., 1990). T7 RNA polymerase use in Gram-positive bacterium was first described by Wells and colleagues (1993) by expressing tetanus toxin fragment C in *L. lactis*.

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4 Publication I

Tuning constitutive recombinant gene expression in *Lactobacillus plantarum*

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MICROBIAL CELL FACTORIES

RESEARCH Open Access

Tuning constitutive recombinant gene expression in *Lactobacillus plantarum*

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Abstract

Background: *Lactobacillus plantarum* constitutes a well-recognized food-grade system for the expression of recombinant proteins in the field of industrial and medical biotechnology. For applications in vivo or in biotechnological processes, the level of expression of e.g. antigens or enzymes is often critical, as expression levels should be of a certain effectiveness, yet, without putting too much strain to the overall system. The key factors that control gene expression are promoter strength, gene copy number and translation efficiency. In order to estimate the impact of these adjusting screws in *L. plantarum* CD033, we have tested several constitutive promoters in combination with high and low copy number plasmid backbones and varying space between the Shine-Dalgarno sequence and the start-codon.

Results: By combining strong promoters, such as transcription elongation factor promoters, isolated from *L. plantarum* CD033 and *L. buchneri* CD034, a synthetic promoter, originally derived from *L. plantarum* WCSF1 and a heterologous promoter derived from *L. buchneri* CD034 with a high and a low copy number origin of replication we demonstrated various expression levels of the model protein mCherry. All promoters were feasible for protein expression and in all cases, the high copy number origin of replication increased expression twofold. We found that the optimal spacer between the Shine-Dalgarno sequence and the start codon in *L. plantarum* consists of 8 nucleotides and elongation as well as shortening this sequence gradually down-regulates gene expression.

Conclusions: We have evaluated the effects of a set of gene regulatory tools to fine tune recombinant gene expression in *L. plantarum* CD033. We have thus, provided potential expression vectors useful for constitutive protein expression in lactic acid bacteria ranging from moderate to strong production levels.

Keywords: *Lactobacillus plantarum* CD033, *Lactobacillus buchneri* CD034, Constitutive promoter, Promoter strength, Elongation factor Tu, Ribosomal binding site, BioLector™

Background

Lactic acid bacteria (LAB) are responsible for various fermentation processes leading to food and feed preservation and improvement in flavour and texture of the fermented substrate [1]. Furthermore, many LAB have been found to be beneficial intestinal microbes associated with human and animal health [2]. Thus, LAB constitute an attractive tool for many applications in food and feed production [3-5], biotechnology [6-9] and medicine [10,11]. Besides using various wild-type LAB, the possibility to expand the genetic repertoire of beneficial strains by genetic engineering becomes more and more attractive. Today, different gene expression systems are available for LAB, many of them optimized for *Lactococcus lactis* [12-15]. Inducible systems allow gene regulation by different additives such as lactose, xylose or other changing parameters like pH or temperature [16]. Another inducible expression system is based on the bacteriocin operon of *Lactobacillus sakei* which was shown to drive high-level gene expression in *L. sakei* and *Lactobacillus plantarum* [17]. Another bacteriocin induced system is the so called NICE-system (nisin-controlled gene expression system, for review see Mierau and Kleerezebem [18]), which was also adapted for use in *L. plantarum* [19]. Inducible expression systems are important when aiming at the overproduction of proteins to a maximum level, when proteins are toxic, or interfere in some other way with the host's metabolism. LAB comprise a food grade background that by genetic engineering may be equipped with additional enzymatic activities that would be beneficial during the process of food and feed

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fermentation [20], for the production of food additives [6,7,21] or in the intestinal environment [22]. For these applications, inducible expression is not feasible; instead, constitutive promoters providing expression of a target gene at a suitable level are desirable. For example, the homologous lactate dehydrogenase promoter was recently used to constitutively express oxalate decarboxylase in *L. plantarum* WCFS1 [22]. It was shown in different studies, that although, bacterial promoters share similar features, promoter strength is strain and context specific and can vary significantly within LAB [23,24]. Therefore, it is necessary to identify and characterize promoters and regulatory sequences for each new host.

Besides promoter activity, also plasmid copy numbers have a major impact on recombinant protein expression. Most of the commonly used plasmid backbones are based on low copy number origins of replication (p256) [17] or high copy number origins of replication (pSH71) [25]. While in the case of ß-glucuronidase expression, a high copy number plasmid lead to increased expression as compared to using a low copy number plasmid, for a second reporter protein, aminopeptidase N, no such effect could be achieved [17]. This phenomenon was explained by gene specific properties and should be taken into consideration. Plasmid copy numbers were determined and estimated to be around three for p256 and 200 for pSH71. Another high copy number plasmid (pCD034-1) was isolated from a *Lactobacillus buchneri* strain, and its origin of replication was shown to support plasmid maintenance in *L. plantarum* [26]. The relative copy number of a pCD034-1-derivative, pCDLbu-1, was estimated to exceed 200 copies per chromosome [27]. Besides transcription and gene dosage, also translation can be a major bottle neck and design of the ribosomal binding site strongly influences protein production levels [28,29].

The goal of this study was to identify and evaluate simple tools and measures to fine-tune recombinant protein expression in *L. plantarum* with the purpose to provide suitable constitutive systems for applications in e.g. feed silage, food fermentation or in vivo drug delivery. Therefore, we compared different autologous and heterologous promoters, the impact of high and low copy number plasmid backbones and the influence of the distance between the Shine-Dalgarno sequence and the translation start signal. Our expression host was *L. plantarum* CD033. This strain has been isolated from a grass silage in Austria and may be used as an efficient starter culture for this purpose. In addition, *L. plantarum* CD033 was previously described to be feasible for highly efficient transformation with non-methylated DNA, allowing direct transfer of a ligation mix or assembled PCR fragments [30]. Therefore, intermediate hosts such as *L. lactis* or *E. coli* for high yield plasmid production are no longer required, which allows us very fast plasmid construction and manipulation, ideal for testing a large set of genetic elements.

Results and discussion

Comparison of promoter activities: vector design

Since no amplification of shuttle vectors in *E. coli* was required, all plasmids were designed without any additional *E. coli* specific origin of replication or selection markers. In the first experiment, we included four different constitutive promoters and tested for cytoplasmic expression of the reporter gene mCherry. The strong P_{11} promoter, a synthetic sequence based on an rRNA promoter from *L. plantarum* WCSF1 [31] was previously shown to be one of the strongest promoters, active in *L. plantarum* as its transcriptional activity was comparable to the inducible pSIP-based expression system [32]. Another beneficial feature of this promoter is its cross species activity, which has been shown for *L. sakei* [31]. Further, the promoter regions upstream of the gene encoding the putative translation elongation factor TU (P_{tuf}) from *L. plantarum* CD033 (P_{tuf33}) and from *L. buchneri* CD034 (P_{tuf34}, this study, see Figure 1) were isolated and tested. Based on the fact that transcription elongation factors are among the most abundant proteins in bacteria, our assumption was that the corresponding promoters would induce strong transcription. We compared P_{tuf}-promoters from two different species in order to evaluate the versatility of this type of promoters and the feasibility for making shuttle vectors between *L. plantarum* and *L. buchneri*. In addition, using a heterologous P_{tuf}-promoter would minimize possible impact by species specific regulation mechanisms. The upstream sequences of the putative elongation factor P (P_{efp}, this study, Figure 2) was isolated from *L. buchneri* CD034 and based on previous observations was identified as quite active in the context of *L. buchneri* (data not shown).

Characterization of selected promoter active fragments

Pretesting of the promotor activities was accomplished by monitoring fluorescence signals of *L. plantarum* CD033 cells carrying the pCD256ΔEc-based constructs using a Tecan™ reader. Cells were cultivated and measurements were performed for 23 h (Figure 3). While the promoter P_{efp} was very weak, good expression could be achieved with both P_{tuf} promoters and the P_{11} promoter. The P_{efp} promoter has previously been tested in *L. buchneri* CD034 and showed medium to high expression of mCherry (data not shown), indicating that its low activity is a species specific effect, and in the context of *L. plantarum* this promoter is not feasible for further experiments.

Impact of plasmid copy number

It has been shown for several plasmid based expression systems, that gene copy numbers have a strong influence on

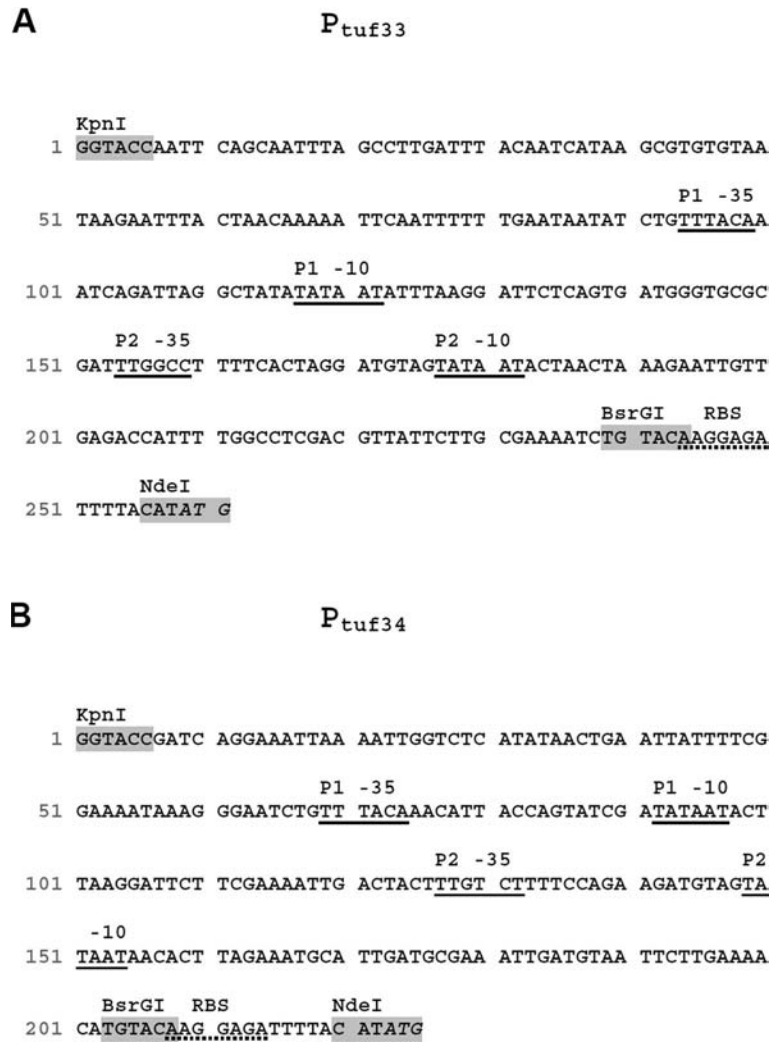

Figure 1 Nucleotide sequences of promoters P_{tuf33} (A) and P_{tuf34} (B). Both promoters are putative tandem promoters, each consisting of two consecutive promoter regions P1 and P2. The −35 and −10 regions are underlined, RBSs are underlined dotted, translation start signals are written in italics, restriction sites are highlighted in gray.

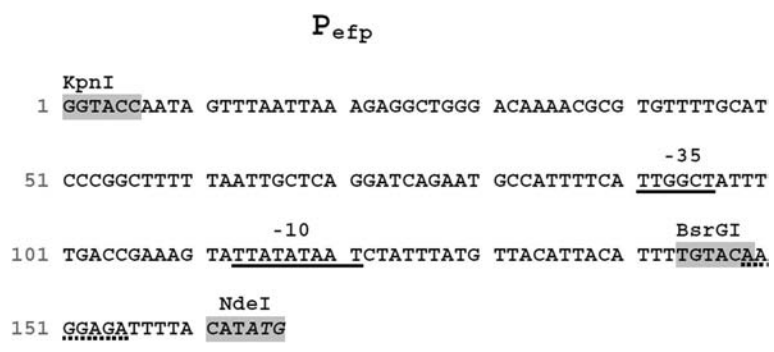

Figure 2 Nucleotide sequence of promoter P_{efp}. Ribosomal binding site, −35 and −10 regions are underlined, translation start signal is written in italics, restriction sites are highlighted in gray.

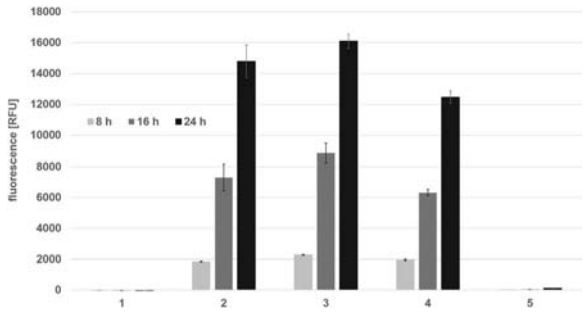

Figure 3 Tecan™ reader measurement of mCherry expression levels in *L. plantarum* CD033 carrying the pCD256ΔEc_mCherry vectors after 8, 16 and 23 hours. Error bars show standard deviation. (1) *L. plantarum* CD033 negative control pCD256ΔEc_mCherry, (2) *L. plantarum* CD033 pCD256ΔEc_Ptuf33_mCherry, (3) *L. plantarum* CD033 pCD256ΔEc_Ptuf34_mCherry, (4) *L. plantarum* CD033 pCD256ΔEc_P11_mCherry, (5) *L. plantarum* CD033 pCD256ΔEc_Pefp_mCherry.

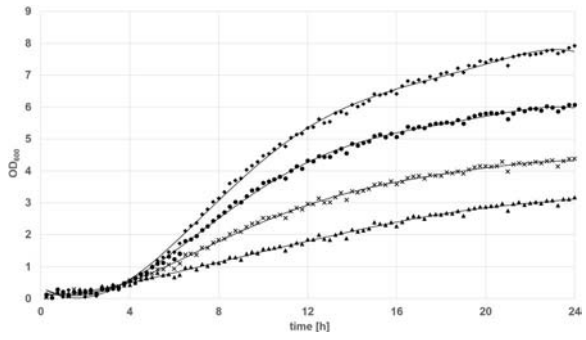

Figure 4 Growth rates of *L. plantarum* CD033 carrying the pCDLbu-1ΔEc_mCherry vectors determined with the BioLector™. (♦) *L. plantarum* CD033 negative control pCDLbu-1ΔEc_mCherry, (●) *L. plantarum* CD033 pCDLbu-1ΔEc_P11_mCherry, (×) *L. plantarum* CD033 pCDLbu-1ΔEc_ Ptuf34_mCherry, (▲) *L. plantarum* CD033 pCDLbu-1ΔEc_ Ptuf33_mCherry.

overall expression of a heterologous protein. While normally an increase leads to higher expression rates, sometimes too high replication rates can be detrimental to cell growth [33]. Most of the theta- or rolling circle replicating plasmids normally used in *L. plantarum* strains have copy numbers between one and five. Yet for the pSIP411-based expression system also the high copy number origin of replication derived from pSH71 is used [17]. In order to investigate the influence of plasmid copy number on the expression level, we constructed several plasmids, either containing the theta replicating, low copy number miniori p256 resulting in pCD256ΔEc plasmid constructs, or the high copy number ori pCD034-1, isolated from *L. buchneri* CD034 [26] resulting in the plasmid pCDLbu-1ΔEc. Table 1 lists all constructs used for fermentation experiments using the BioLector™ platform.

When looking at the growth rates of the tested clones (Figure 4), it becomes apparent that pCDLbu-1ΔEc _P_{tuf33}_mCherry and pCDLbu-1ΔEc_P_{tuf34}_mCherry, both containing the high copy number origin of replication, produce less biomass during fermentation. This might be because, due to overproduction of mCherry, the overall metabolic load hampers the growth rate. Alternatively, the high number of P_{tuf}-promoter copies may capture essential sigma factors, and the cell is unable to proceed with translation of homologous genes at a normal rate. When comparing the overall transcriptional activities (Figure 5), the P_{11} promoter in combination with the high copy number plasmid backbone turned out to be the strongest, followed by the two other pCDLbu-1ΔEc-based constructs. All fermentations based on the theta replicating plasmids produced about half the fluorescence signal as compared to their rolling circle replicating counterpart (Figure 6). Growth rates were comparable for all pCD256ΔEc-based constructs (data not shown). Specific gene expression as shown in Figure 7A reflects the impact of growth inhibition in case of the high copy number constructs, while for the low copy number plasmids, specific expression rates were comparable (Figure 7B).

Table 1 Description of constructs indicating the promoter and origin of replication present on each plasmid

Construct	Promoter	Promoter from	Reference	Origin of replication from
pCDLbu-1ΔEc_mCherry (neg. contr.)	None	w/o promoter	This study	pCD034-1
pCD256ΔEc_mCherry (neg. contr.)	None	w/o promoter	This study	p256
pCDLbu-1ΔEc_Pefp_mCherry	P_{efp}	*L. buchneri* CD034	This study	pCD034-1
pCD256ΔEc_Pefp_mCherry	P_{efp}	*L. buchneri* CD034	This study	p256
pCDLbu-1ΔEc_Ptuf33_mCherry	P_{tuf33}	*L. plantarum* CD033	This study	pCD034-1
pCD256ΔEc_Ptuf33_mCherry	P_{tuf33}	*L. plantarum* CD033	This study	p256
pCDLbu-1ΔEc_Ptuf34_mCherry	P_{tuf34}	*L. buchneri* CD034	This study	pCD034-1
pCD256ΔEc_Ptuf34_mCherry	P_{tuf34}	*L. buchneri* CD034	This study	p256
pCDLbu-1ΔEc_P11_mCherry	P_{11}	*L. plantarum* library	[31]	pCD034-1
pCD256ΔEc_P11_mCherry	P_{11}	*L. plantarum* library	[31]	p256

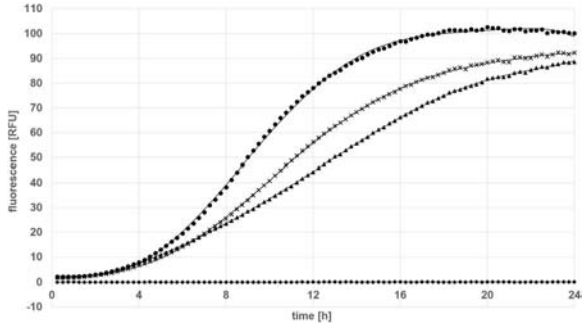

Figure 5 BioLector™ analysis of mCherry expression levels in *L. plantarum* CD033 carrying the pCDLbu-1ΔEc_mCherry constructs. (●) *L. plantarum* CD033 pCDLbu-1ΔEc_P11_mCherry, (×) *L. plantarum* CD033 pCDLbu-1ΔEc_ Ptuf34_mCherry, (▲) *L. plantarum* CD033 pCDLbu-1ΔEc_ Ptuf33_mCherry, (♦) *L. plantarum* CD033 negative control pCDLbu-1ΔEc_mCherry.

Influence of the relative position of the Shine-Dalgarno sequence

The specific base pairing between the 3′-end of the rRNA and the sequence preceding an initiator AUG provides a mechanism by which the cell can distinguish between initiator AUGs and internal and/or out-of-phase AUG sequences. The degree of base pairing also plays a role in determining the rate of initiation at different AUG initiator codons in polycistronic mRNAs [34]. The ribosomal binding site (RBS) or Shine-Dalgarno-sequence (SDS) used in our expression constructs was originally derived from pSIP409. This sequence, AAGGAGA [31], however, did not correspond to the perfect matching SDS core sequence AAGGAGG, identified in *Lactobacillus plantarum*. Therefore, the RBS of the *slpB* gene from *L. buchneri* CD034, which fits better to the SDS core sequence and corresponds to the most abundantly expressed gene in *L. buchneri* CD034 was chosen for RBS-optimization (Table 2, SDOPT#9). The distance between the SDS and the start codon of our constructs comprised 9 nucleotides. For fine-tuning translational efficiency we changed the SDS to the perfect match sequence and varied the distance between the SDS and the translational start-site, analysing the range between 5 and 12 nucleotides (Table 2). All constructs were based on the low copy p256 origin of replication and mCherry expression was under control of the P_{11} promoter. The low copy origin of replication was chosen for these experiments in order to provide expression levels that can be up-regulated without causing growth hampering stress due to over-production.

Growth rates were comparable for all constructs (data not shown). Figure 8 shows the correlation between differences in fluorescent signals and the varied length of the spacer sequences. The highest expression was detected after 18 h of cultivation. Spacer sequences shorter than 7 nucleotides turned out to considerably hamper

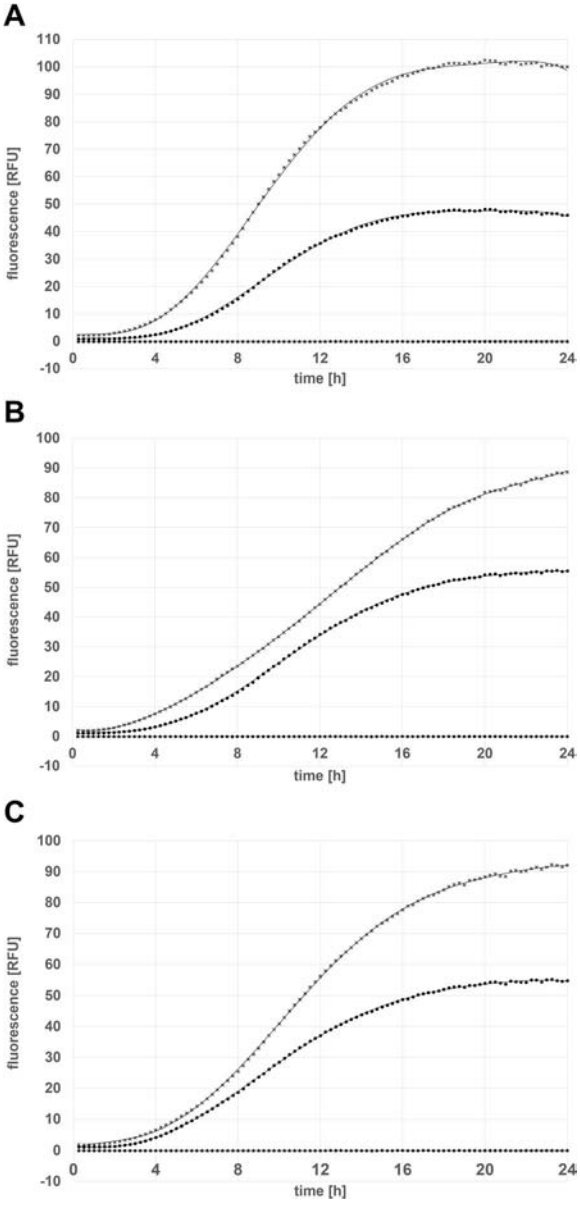

Figure 6 Comparison of mCherry expression levels for the constructs pCDLbu_1ΔEc and pCD256ΔEc in *L. plantarum* CD033 measured using BioLector™ platform: mCherry under control of promoter (A) P11: (♦) *L. plantarum* CD033 negative control pCDLbu-1ΔEc_mCherry, (▲) *L. plantarum* CD033 negative control pCD256ΔEc_mCherry, (×) *L. plantarum* CD033 pCDL bu-1ΔEc_P11_mCherry, (●) *L. plantarum* CD033 pCD256Δ Ec_P11_mCherry **(B)** P_{tuf33}: (♦) *L. plantarum* CD033 negative control pCDLbu-1ΔEc_mCherry, (▲) *L. plantarum* CD033 negative control pCD256ΔEc_mCherry, (×) *L. plantarum* CD033 pCDL bu-1ΔEc_Ptuf33_mCherry, (●) *L. plantarum* CD033 pCD256Δ Ec_Ptuf33_mCherry and under control of promoter **(C)** P_{tuf34}: (♦) *L. plantarum* CD033 negative control pCDLbu-1ΔEc_mCherry, (▲) *L. plantarum* CD033 negative control pCD256ΔEc_mCherry, (×) *L. plantarum* CD033 pCDLbu-1ΔEc_Ptuf34_mCherry, (●) *L. plantarum* CD033 pCD256ΔEc_Ptuf34_mCherry.

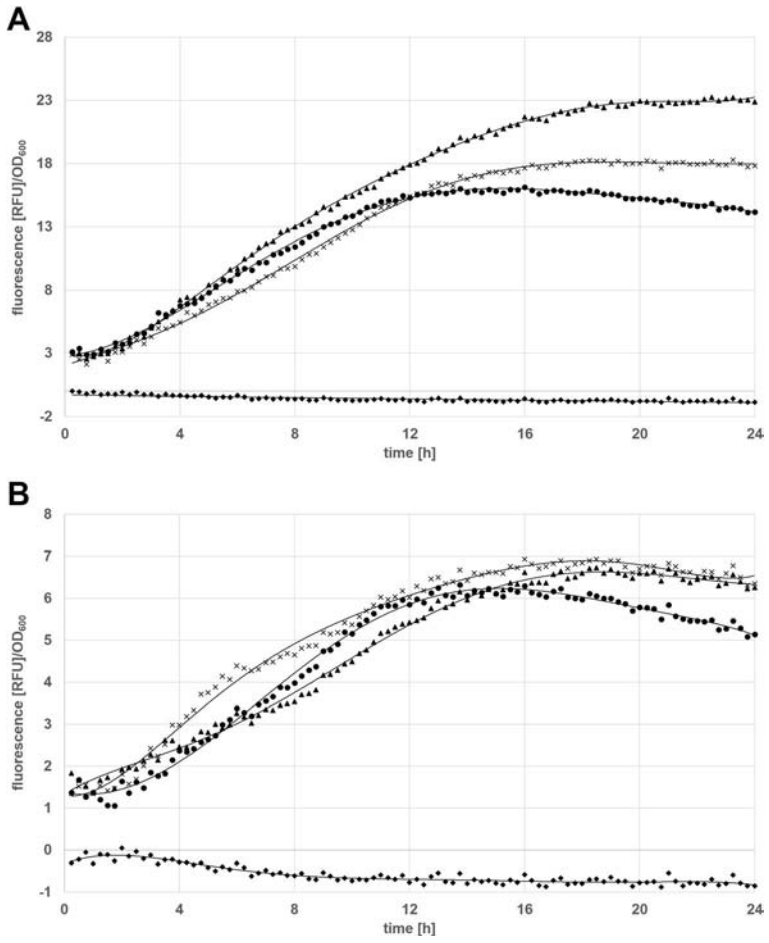

Figure 7 Specific expression rates of the pCDLbu_1ΔEc and pCD256ΔEc constructs in *L. plantarum* CD033 determined with the BioLector™. (A) pCDLbu_1ΔEc constructs: (▲) *L. plantarum* CD033 pCDLbu-1ΔEc_ Ptuf33_mCherry, (×) *L. plantarum* CD033 pCDLbu-1ΔEc_Ptuf34_mCherry, (●) *L. plantarum* CD033 pCDLbu-1ΔEc_P11_mCherry (♦) *L. plantarum* CD033 negative control pCDLbu-1ΔEc_mCherry **(B)** pCD256ΔEc constructs: (×) *L. plantarum* CD033 pCD256ΔEc_Ptuf34_mCherry, (▲) *L. plantarum* CD033 pCD256ΔEc_Ptuf33_mCherry, (●) *L. plantarum* CD033 pCD256ΔEc_P11_mCherry, (♦) *L. plantarum* CD033 negative control pCD256ΔEc_mCherry.

Table 2 List of constructs with varying spacer sequences between the SDS and the start codon

Construct	SDS-spacer-start codon
SDOPT#5	AAGGAGG **AATAC** ATG
SDOPT#6	AAGGAGG **AAATAC** ATG
SDOPT#7	AAGGAGG **AAATTAC** ATG
SDOPT#8	AAGGAGG **AAATTTAC** ATG
SDOPT#9	AAGGAGG **AAATTATAC** ATG
SDOPT#10	AAGGAGG **AAAATTATAC** ATG
SDOPT#11	AAGGAGG **AAAAATTATAC** ATG
SDOPT#12	AAGGAGG **AAAAAATTATAC** ATG

Spacer sequences are written in bold.

translation efficiency, while 8 nucleotides seemed to be optimal. A slight decrease could be observed when the spacer was designed to be as long as 12 nucleotides. Thus, if desired, recombinant expression may be down-regulated by using spacer variations shorter than 7 or longer than 11 nucleotides. Fine-tuning of protein expression in order to utilize a host cell in an optimal way can be realized by regulation of several parameters. Here, we investigated and demonstrated the impact of transcriptional activity, gene copy number and translation efficiency for the species *Lactobacillus plantarum*. Considering that many strains of this species are used as highly beneficial starter cultures for food and feed applications, the potential applications are manifold. Over-expression of cellulases and hemicellulases could contribute to digestibility and quality of grass silage. Moreover, new substrates, such as leaves or other plant waste material could be fermented more efficiently and fed into biogas plants.

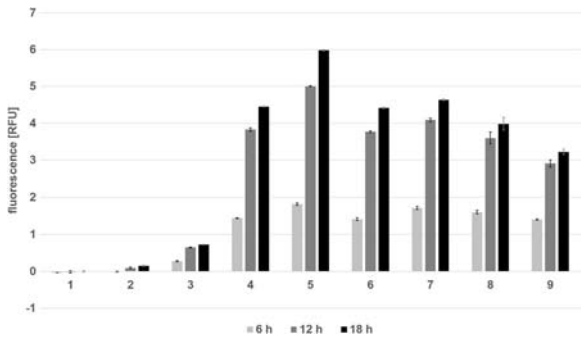

Figure 8 BioLector™ analysis of the SDS-varied pCD256ΔE c_mCherry constructs after 6, 12 and 18 hours. Error bars show standard deviation. (1) *L. plantarum* CD033 negative control pCD256ΔEc_mCherry, (2) *L. plantarum* CD033 pCD256ΔE c_P11_mCherry SDOPT#5, (3) *L. plantarum* CD033 pCD256ΔE c_P11_mCherry SDOPT#6, (4) *L. plantarum* CD033 pCD256Δ Ec_P11_mCherry SDOPT#7, (5) *L. plantarum* CD033 pCD256Δ Ec_P11_mCherry SDOPT#8, (6) *L. plantarum* CD033 pCD25 6ΔExc_P11_mCherry SDOPT#9, (7) *L. plantarum* CD033 pCD25 6ΔEc_P11_mCherry SDOPT#10, (8) *L. plantarum* CD033 pCD25 6ΔEc_P11_mCherry SDOPT#11, (9) *L. plantarum* CD033 pCD25 6ΔEc_P11_mCherry SDOPT#12.

Another application is the food-grade expression of enzymes for making nutritional additives, e.g. ß-galactosidase or chitinase are used to produce oligo-saccharides [6,7]. Finally, *L. plantarum* is a widely spread probiotic and as such may be used as a scaffold for vaccination or treatment of intestinal diseases in vivo. Next steps are to test different strains within the species of *L. plantarum* as well as different genes with biotechnological potential.

Conclusions

Lactobacillus plantarum is widely spread in nature. It is used as a highly effective silage additive, has probiotic properties and serves as a cell factory to produce recombinant proteins. Here we have tested several constitutive promoters in combination with high and low copy number plasmid backbones in *L. plantarum* CD033. Thereby, we confirmed the previously described promoter P_{11} [31] to be feasible for strong constitutive protein expression, especially in combination with a high copy number origin of replication. We further characterized two new promoters, P_{tuf33} and P_{tuf34}, which now are available as additional candidates to drive constitutive expression in *L. plantarum* as well as in *L. buchneri*. The impact of different origins of replication was investigated, demonstrating twofold higher product yields for the pCDLbu-1ΔEc-based constructs containing the high copy number origin of replication derived from the *L. buchneri* CD034 plasmid pCD034-1 [26]. Besides controlling transcriptional levels and gene copy number, we evaluated the possibility to up or down-regulate the overall target gene expression by varying the distance between the SDS and the start-codon. We could show that in *L. plantarum* CD033 there was a direct correlation between these two parameters, reaching the highest expression levels when the spacer spanned 8 nucleotides. While the performance and behavior of expression regulatory elements might differ in dependence of the target gene, predictions about their impact will facilitate vector design strategies and experimental set-ups in the future. Overall, we believe that the silage strain *L. plantarum* CD033 as well as the *L. plantarum* species in general is a highly versatile tool for improving nutrition quality, human health and biomass based energy production.

Methods

Cultivation and transformation of *L. plantarum* CD033

The *Lactobacillus plantarum* strain CD033 was grown in de Man-Rogosa-Sharpe (MRS) medium [35] at 30°C under oxygen limitation, supplemented with chloramphenicol (10 μg ml^{-1}) if required. The transformation of plasmids into *L. plantarum* CD033 was accomplished according to the electroporation protocol described earlier [27].

DNA techniques and cloning procedure

All Enzymes were purchased from New England Biolabs (NEB, USA). DNA fragments were amplified using the Phusion High-Fidelity DNA Polymerase according to the manufacturer's recommendations. All resulting clones were colony screened using OneTaq DNA Polymerase as recommended by the producer. All PCRs were carried out with a C1000 Thermal Cycler (BioRad, USA). Restriction digests were performed following the manufacturer's instructions. PCR products were purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Germany). Ligations were performed using T4-ligase. All primers are listed in Table 3.

Construction of expression vectors for promoter activity testing

A gene, codon optimized for *L. plantarum* WCFS1 using the webtool JCat (http://www.jcat.de/), encoding the red fluorescent protein mCherry was synthesized as a gBlock (IDT, Belgium) and amplified using the primers mCherry_F (NdeI)/mCherry_R (BamHI). Promotor P_{11} was also amplified from a gBlock using the primers P11_F (SacI, KpnI)/P11_R (NdeI). The two PCR products were digested with NdeI and ligated one with each other to gain the DNA-fragment P11_mCherry. The ligation product was again amplified using the primers P11_F (SacI,KpnI)/mCherry_R (BamHI).

Theta-replicating pCD256ΔEc-constructs

For construction of the theta-replicating expression vectors the plasmid pCD256ΔEC_hTTF1 [30] was amplified using the primers sCAT_R (KpnI)/Tldh_F (BamHI). The PCR

Table 3 List of primers used in this study

Primer	Sequence 5′->3′
mCherry_F (NdeI)	CAGCAGCAG**CATATG**TTATCAAAGGGTGAAGAAG
mCherry_R (BamHI)	CGTCGT**GGATCC**TTATCACTTGTATAATTCATCCATACC
P11_F (SacI,KpnI)	GACGAC**GAGCTCGGTACC**TTACAGCTCCAGATCTAGCG
P11_R (NdeI)	GACGACGAC**CATATG**TAAAATCTCCTTGTAATAGTATTT
sCAT_R (KpnI)	GCTGCT**GGTACC**GGGCAGGTTAGTGACATTAG
Tldh_F (BamHI)	CTGCTG**GGATCC**AAAACCGCTGT
mCherry_F (BsrGI)	CAGCAG**TGTACA**AGGAGATTTTACATATGTTATCAAAGGGTGAAG
Ptuf_CD033_F (KpnI)	GACGAC**GGTACC**AATTCAGCAATTTAGCCTTGATTTAC
Ptuf33_R (BsrGI)	GTCCAG**TGTACA**GATTTTCGCAAGAATAACGTCG
Ptuf_CD034_F (KpnI)	GTCGTC**GGTACC**GATCAGGAAATTAAAATTGGTCTC
Ptuf34_R (BsrGI)	GTCGTC**TGTACA**TGTTTTCAAGAATTACATCAATTTCG
efp-sense_F (MfeI, KpnI)	GCAGCA**CAATTGGGTACC**AATAGTTTAATTAAAGAGGCTGG
Pefp_R (BsrGI)	CAGCAG**TGTACA**AAATGTAATGTAACA
Cat_F (NheI)	CGACGA**GCTAGC**AATGTGGTCTTTATTCTTCAAC
M13_R (NheI)	CGACGA**GCTAGC**AGCCAGGAAACAGCTATGACC
Tldh_amp_R (PstI)	CTGCTG**CTGCAG**AAAAAGATTAAAAAAGCCGCTGC
P11_control_R (KpnI)	CTGCAC**GGTACC**CAAGGAGATTTTACATATGTTATCA
Cat_seq2_back	TACATCATTCTGTTTGTGATGG
4_6_n2_R	AACTCATAATACGCCTAAGCC
EFP_screen_back	GATTCCCGATAACAACCGT
SDOPT_5_F (XbaI)	ACGACG**TCTAGA**TAAGGAGGAATACATGTTATCAAAGGGTGAAGAAG
SDOPT_6_F (XbaI)	ACGACG**TCTAGA**TAAGGAGGAAATACATGTTATCAAAGGGTGAAGAAG
SDOPT_7_F (XbaI)	ACGACG**TCTAGA**TAAGGAGGAAATTACATGTTATCAAAGGGTGAAGAAG
SDOPT_8_F (XbaI)	ACGACG**TCTAGA**TAAGGAGGAAATTTACATGTTATCAAAGGGTGAAGAAG
SDOPT_9_F (XbaI)	ACGACG**TCTAGA**TAAGGAGGAAATTATACATGTTATCAAAGGGTGAAGAAG
SDOPT_10_F (XbaI)	ACGACG**TCTAGA**TAAGGAGGAAAATTATACATGTTATCAAAGGGTGAAGAAG
SDOPT_11_F (XbaI)	ACGACG**TCTAGA**TAAGGAGGAAAAATTATACATGTTATCAAAGGGTGAAGAAG
SDOPT_12_F (XbaI)	ACGACG**TCTAGA**TAAGGAGGAAAAAATTATACATGTTATCAAAGGGTGAAGAAG
SDOPT_R (XbaI)	ACGACG**TCTAGA**GAATACATATATGCTGGCCAGC

Restriction sites are written in bold letters.

product was double digested with restriction endonucleases KpnI and BamHI and fragment P11_mcherry was ligated into the vector backbone. The resulting construct was designated pCD256 ΔEC _P11_mCherry.

To get constructs with the other promoters, pCD256 ΔEC _P11_mcherry was amplified using the primers mCherry_F (BsrGI)/sCAT_R (KpnI). The promoters were amplified using the primers Ptuf_CD033_F (KpnI)/Ptuf33_R (BsrGI) for promoter P_{tuf33}, Ptuf_CD034_F (KpnI)/Ptuf34_R (BsrGI) for promoter P_{tuf34} and efp-sense_F (MfeI, KpnI)/Pefp_R (BsrGI) for the efp-promoter P_{efp}. The PCR products were KpnI/BsrGI digested and each promotor was ligated with the vector backbone described above. The resulting constructs were pCD256ΔEc_Ptuf33_mCherry, pCD256ΔEc_Ptuf34_mCherry and pCD256ΔEc_Pefp_mCherry. All constructs were introduced into *L. plantarum* CD033 by electroporation. The pCD256ΔEc constructs were colony screened using the primers Cat_seq2_back and efp_screen_back. All constructs were confirmed by sequencing using the same primers (Microsynth, Switzerland).

Rolling circle replicating (RCR) pCDLbu-1ΔEc-constructs

For the RCR-constructs plasmid pCDLbu-1 [26] served as vector backbone. First all *E.coli*-specific sequences were removed by PCR using the primers Cat_F (NheI)/M13_R (NheI). After NheI digestion the amplicon was recircularized by selfligation and transformed directly into *L. plantarum* CD033. The resulting vector was designated pCDLbu-1ΔEC and was subsequently amplified

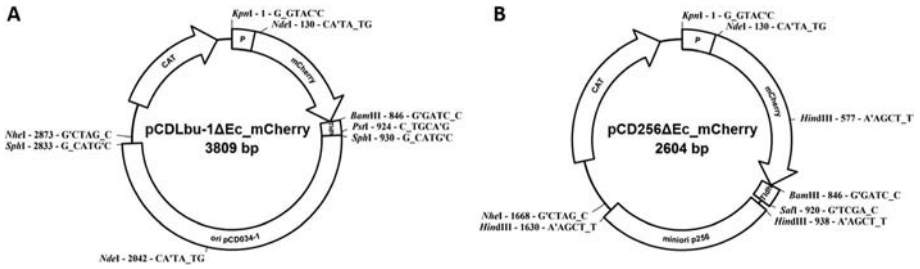

Figure 9 Maps of pCDLbu1ΔEc_mCherry and pCD256ΔEc_mCherry. (A) Map of pCDLbu-1ΔEc_mCherry consisting of the high copy replicon from plasmid pCD034-1 (Heinl et al. 2011), a chloramphenicol resistance gene for selection in LAB, the mCherry expression cassette including one of the chosen promotors, an mCherry reporter gene and the T_{ldh} terminator from *L. casei* (Spath et al. 2012b). **(B)** Map of pCD256ΔEc_mCherry containing the minimal origin of replication from plasmid p256, the chloramphenicol resistance gene, and the mCherry expression cassette.

using the primers sCAT_R (KpnI)/Tldh_F (BamHI) and digested with KpnI/PstI. The already finished pCD256ΔEc vectors served as template for insert amplification. Therefore, forward primers P11_F (SacI/KpnI), efp-sense_F (MfeI,KpnI), Ptuf_CD033_F (KpnI), Ptuf_CD034_F (KpnI) and the reverse primer Tldh_amp_R (PstI) were used to obtain the expression cassettes P11_mCherry_Tldh, Pefp_mCherry_Tldh, Ptuf33_mCherry_Tldh and Ptuf34_mCherry_Tldh. After a KpnI/PstI digest the inserts were ligated with the pCDLbu-1ΔEc backbone to gain the constructs pCDLbu-1ΔEc _P11_mCherry, pCDLbu-1ΔEc_Pefp_mCherry, pCDLbu-1ΔEc_Ptuf33_mCherry and pCDLbu-1ΔEc_Ptuf34_mCherry which were used to transform *L. plantarum* CD033 by electroporation. The pCDLbu1ΔEc constructs were colony screened using the primer pair Cat_seq2_back /4_6_n2_R. All constructs were confirmed by sequencing using the same primers (Microsynth, Switzerland).

The general vector designs are shown in Figure 9.

Cloning of negative controls

Plasmids pCDLbu1ΔEc_mCherry and pCD256ΔEc_mCherry lacking a promoter upstream of the mCherry gene served as negative controls. Therefore, plasmids pCDLbu1-ΔEc _P11_mCherry and the pCD256ΔEc _P11_mCherry were amplified using the primers P11_control_R (KpnI)/ sCAT_R (KpnI). After a KpnI-digest the PCR products were self-ligated and used to transform *L. plantarum* CD033.

Constructs for Shine-Dalgarno Optimization

Plasmid pCD256_P11_mCherry was used as PCR template for this experiment. Constructs were amplified using the forward primers SDOPT_5_F (XbaI), SDOPT_6_F (XbaI), SDOPT_7_F (XbaI), SDOPT_8_F (XbaI) SDOPT_9_F (XbaI), SDOPT_10_F (XbaI), SDOPT_11_F (XbaI) and SDOPT_12_F (XbaI) and the reverse primer SDOPT_R (XbaI). Restriction digests with XbaI were performed. The DNA-fragments were self-ligated and used to transform *L. plantarum* CD033.

Colonies resistant to chloramphenicol were screened by PCR using the primers Cat_seq2_back/EFP_screen_back and correctness of the constructs was confirmed by sequencing of the obtained PCR-products (Microsynth, Switzerland).

Determination of mCherry expression by Tecan™ reader measurement

The Infinite M1000 Tecan™ reader connected to the Tecan i-control 1.6 software (Tecan Group Ltd., Switzerland) was used for pretesting. Overnight cultures were diluted to an OD_{600} value of 0.1. 200 μL of each sample was pipetted into a 96 well clear bottom plate (Perkin Elmer, USA). The mCherry fluorescence at 587 nm was measured at 30°C every 30 minutes over 23 h. A gain of 140 was used for fluorescence measurments. Immediately prior to fluorescence measurment, samples were shaken for 15 seconds. Samples were analyzed in quadruplicate.

Determination of mCherry expression by BioLector™ measurement

mCherry measurements were accomplished using the BioLector™ Basic device (m2p-labs Germany). Data were analyzed using the BioLection 2.3.13 software (m2p-labs, Germany). Overnight cultures were diluted to an OD_{600} value of 0.1 and subsequently 800 μl of each sample were pipetted into a MTP-48 FlowerPlate™ (m2p-labs, Germany). Fluorescence was determined using the E-OP-119 LED module for mCherry at 580 nm and a gain of 80. Measurement was executed every 15 minutes, cells were cultivated at 30°C for 24 hours under constant shaking at 1,000 rpm. Negative controls were analyzed in duplicate, samples were analyzed in triplicate. For biomass analysis a calibration curve was generated. The OD_{600} values of a *L. plantarum* CD033 o/n cultures were measured undiluted, 1:1.3, 1:2, 1:3, 1:4, 1:5, 1:7, 1:10 and 1:20 diluted in an Implen Nano Photometer (Implen, Germany) and correlated with the scattered light data at 620 nm and a gain of 20 measured using the BioLector™ system. The linear equation of the

standard curve was y = 0.0312x – 0.6465 with a correlation coefficient R = 0.9991.

Abbreviations

H: Hours; O/N: Over Night; OD_{600}: Optical density at 600 nm; RCR: Rolling circle replicating; RFU: Relative fluorescence units; RPM: Rounds per minutes; SDS: Shine-Dalgarno sequence; W/O: Without.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

The work presented here was carried out in collaboration between all authors. S Heinl and RG defined the research theme and designed the experiments. CT, EE and S Heiss carried out the laboratory experiments, analyzed the data, interpreted the results and prepared this manuscript with input, feedback and advice from S Heinl and RG. All authors have contributed to, seen and approved the manuscript.

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Publication II

Characterization of the *Lactobacillus plantarum* plasmid pCD033 and generation of the plasmid free strain *L. plantarum* 3NSH

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Method Article

Characterization of the *Lactobacillus plantarum* plasmid pCD033 and generation of the plasmid free strain *L. plantarum* 3NSH

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ABSTRACT

Lactobacillus plantarum CD033, a strain isolated from grass silage in Austria, harbors a 7.9 kb plasmid designated pCD033. Sequence analysis identified 14 open reading frames and 8 of these were supposed to be putative coding sequences. Gene annotation revealed no putative essential genes being plasmid encoded, but a plasmid addiction system based on a PemI/PemK-like toxin–antitoxin system, able to stabilize plasmid maintenance. Absence of a replication initiation protein, a double strand origin as well as a single strand origin on plasmid pCD033 suggests replication via a new type of theta mechanism, whereby plasmid replication is potentially initiated and regulated by non-coding RNA. Detailed examination of segregational stability of plasmid vectors consisting of pCD033-fragments, combined with a selection marker, resulted in definition of a stably maintained minimal replicon. A gene encoding a RepB/OrfX-like protein was found to be not essential for plasmid replication. Alignment of the amino acid sequence of this protein with related proteins unveiled a highly conserved amino acid motif (LLDQQQ). *L. plantarum* CD033 was cured of pCD033 resulting in the novel plasmid free strain *L. plantarum* 3NSH. Plasmid curing demonstrated that no essential features are provided by pCD033 under laboratory conditions.

1. Introduction

Lactobacillus plantarum CD033 is a lactic acid bacterium (LAB) that was isolated from grass silage and was previously characterized regarding its use as a possible expression host (Spath et al., 2012a,2012b). The species *L. plantarum* belongs to the mammalian oral- and gastrointestinal natural inhabitants (Kleerebezem et al., 2003), is responsible for fermentation of meat and milk products as well as for preservation of feed raw material such as grass and cereal grains (Wanker et al., 1995; Siezen et al., 2010). Since LAB have a long history in food and feed fermentations they are generally regarded as safe (GRAS) organisms (Douillard and de Vos, 2014). Besides their ability to preserve food by acidification, they often produce antimicrobial peptides called bacteriocins (Wang and Lee, 1997). For instance, *L. plantarum* was found to produce plantaricin, which was shown to enhance feed preservation and to exhibit probiotic effects in farm animals (Muñoz et al., 2011).

The presence of small circular plasmids is quite common in *L. plantarum* (Cho et al., 2013; Jalilsood et al., 2014; Zhang et al., 2015). Specific phenotypes such as the production of bacteriocins, resistance to antibiotics and metabolism of carbohydrates are often plasmid-encoded (Wang and Lee, 1997). Sometimes, no obvious function can be assigned to plasmids, yet, these so called 'cryptic plasmids' are stably maintained within their host cells.

Several whole genomes of *L. plantarum* species were sequenced in the last decade and show diverse distribution of plasmid numbers per cell and replication modes. Reports describe naturally plasmid free strains (Axelsson et al., 2012),

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strains containing cryptic plasmids without obvious importance to the cell and strains with up to eight different plasmids, some of which encode essential features (Crowley et al., 2013). It is known that small multi-copy plasmids of lactobacilli mostly replicate by the rolling circle mode, but also theta replication is quite common (del Solar et al., 1998). Both these replication mechanisms generally require a replication protein for initiation of replication. Based on these two replication modes, some shuttle vectors have been established for recombinant protein expression in lactobacilli. In general, theta replicating plasmids are known to be segregationally and structurally more stable since no single stranded DNA intermediates occur during the replication process. Applications in recombinant protein technology of LAB strongly rely on the development of stable cloning vectors derived from plasmids with GRAS origins (Shareck et al., 2004).

L. plantarum CD033 was found to contain one plasmid with a size of 7.9 kb. In our study we characterized the plasmid (designated pCD033) in terms of copy number, segregational stability, and definition of a minimal stable replicon. Lack of a replication protein, of a double strand origin as well as of a single strand origin suggests a novel mode of replication. Successful curing indicated that pCD033 is not essential for *L. plantarum* CD033. In addition, growth rate and robustness was not impaired after plasmid curing. Characterization of pCD033 and the knowledge about its replication mechanism provides a new tool for the development of novel shuttle vectors and stable recombinant protein expression in *L. plantarum*.

2. Materials and methods

2.1. Bacterial strains, culture media and growth conditions

Escherichia coli JM109 was grown on Lysogeny Broth (LB) (Bertani, 1951) supplemented with 100 μg ml^{-1} ampicillin for selection of recombinant clones. *L. plantarum* CD033 was isolated from a stable grass silage in Austria (Spath et al., 2012a) and *L. plantarum* 3NSH was generated thereof by plasmid curing (this study). All LAB strains were cultivated in De Man–Rogosa–Sharpe (MRS) medium at 30 °C (De Man et al., 1960) without shaking. *L. plantarum* transformants were selected on MRS medium containing 5 or 10 μg ml^{-1} chloramphenicol (CM).

Table 1
List of primers used. Restriction sites are highlighted in gray.

Primer	Sequence 5'→3'
M13F	TGTAAAACGACGGCCAGT
M13R	CAGGAAACAGCTATGACC
PCN268F	TTCTGGATTATCTGACGCACG
PCN268R	TACTTCTCCACTCTATAGCCG
cbhF	TGACTGCCATAACTTATCAATC
cbhR	TCTCCACCTTACGAAATACTAG
pCD033_ori1_F_*Eco*RI	GTAGGAATTCTAGCAGCTTTCG
pCD033_ori2_F_*Eco*RI	GTAGGAATTCAATGCTTGCTGATCCTTGGC
pCD033_ori3_F_*Eco*RI	GTAGGAATTCTCAATGGTAGCGTGGGAGC
pCD033_ori4_F_*Eco*RI	ACAGATGAATTCAATGTTACAGGCTGTTCTATAG
pCD033_ori1_R_*Kas*I	GTAGTAGGCGCCAGATGGGTTTGTGGTAAACAC
pCD033_ori2_R_*Kas*I	GTAGTAGGCGCCATACCCCTTTGTCATATTATCG
CAT_F_*Kas*I	CATCATGGCGCCCTCGCCGGCAATAGTTACC
CAT_R_*Eco*RI	GCTGCTGAATTCGGGCAGGTTAGTGACATTAG
CAT_R_*Kas*I	GCTGCTGGCGCCGGGCAGGTTAGTGACATTAG
CAT_F_*Pst*I	CATCATCTGCAGCTCGCCGGCAATAGTTACC
pCD033_ori1_R_*Kas*I	GTAGTAGGCGCCAGATGGGTTTGTGGTAAACAC
pemI_R_*Pst*I	CAGCAGCTGCAGCTTACCATTGAAGTTCATTACC
repB_probe_F	GATCAATTAGATAAAATGCAGTC
ori_pCD033_probe_R	GTGACGATTAGTACATTAATAAC
cbh_probe_F	TCTAAATATTATTTTCGAGGAGG
cbh_probe_R	AACTGATAACAAACTGATTTCC

2.2. DNA manipulation

Total DNA was extracted using the DNeasy Blood & Tissue Kit (QIAGEN). DNA constructs were generated by using standard laboratory protocols (Sambrook and Russel, 2001). The NucleoSpin® Plasmid QuickPure kit (Macherey-Nagel, Germany) was used with addition of lysozyme (Sigma-Aldrich, Germany) in the resuspension buffer for plasmid extraction. PCR fragments were purified using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Germany). All primers were purchased from IDT (Integrated DNA Technologies, Belgium). Q5® High-Fidelity DNA polymerase, all restriction enzymes, T4 DNA ligase and Antarctic phosphatase were purchased from New England Biolabs (NEB, USA). KOD DNA-polymerase was obtained from Novagen (Toyoba, Japan).

2.3. Transformation of bacteria

Transformation of *E. coli* JM109 cells was carried out by electroporation according to Sambrook and Russel (2001). Transformation of *L. plantarum* was performed by electroporation as described elsewhere (Spath et al., 2012a).

2.4. Plasmid isolation, cloning and sequencing

Plasmid pCD033 was isolated from *L. plantarum* CD033 by a modified miniprep protocol employing 25 μg ml^{-1} lysozyme in the resuspension buffer and incubated for 1 h at 37 °C previous to cell lysis. Plasmid DNA was subjected to agarose gel electrophoresis. A single visible plasmid band was excised and extracted. Purified plasmid DNA was digested with *Sau*3A. Restriction fragments were ligated into a *Bam*HI digested and dephosphorylated pUC19 vector (Invitrogen, USA) transformed into *E. coli* JM109 competent cells. Clones of interest were sequenced by Sanger method (MWG, Ebersberg, Germany) using primers M13F and M13R (Table 1). The whole sequence was finally elucidated by primer walking on one large amplicon generated by PCR using KOD DNA-polymerase (walking primers are not stated).

2.4.1. Sequence analysis and gene annotation

Sequence contigs were assembled using SeqMan software (DNASTAR, USA). Open reading frames and putative coding sequences were identified using pDRAW32 software (http://www.acaclone.com/), adjusted to following parameters: minimal length: 200, genetic code: bacterial as well as plant plastid code and alternative initiation codons: GTG, TTG, ATT & CTG. Using these settings, coding sequences were predicted by manual annotation. Sequence comparison and gene function assignment was completed by BLAST analysis (Altschul and Lipman, 1990). BPROM (http://www.softberry.com) was used to identify σ70-promoters (Solovyev and Salamov, 2011), rho-independent transcriptional terminator prediction was performed with ARNold (http://rna.igmors.u-psud.fr/toolbox/arnold/). Direct and inverted repeats were identified by using EMBOSS (Rice et al., 2000). The plasmid map (Fig. 1) indicates the positions of putative coding sequences (CDSs).

2.5. Plasmid copy number determination of pCD033

Relative plasmid copy number (PCN) was determined by quantitative PCR. Total DNA of *L. plantarum* CD033 cells (OD_{600} of 1) of two samples was prepared by phenol/chloroform extraction. Three replicates in five-fold serial dilutions were analyzed in a CFX96 MiniOpticon real-time PCR detection system (BioRad) with the primers PCN268F, PCN268R, cbhF and cbhR (250 nM each forward and reverse, Table 1) in triplicates. Relative values were measured in 5-fold serial

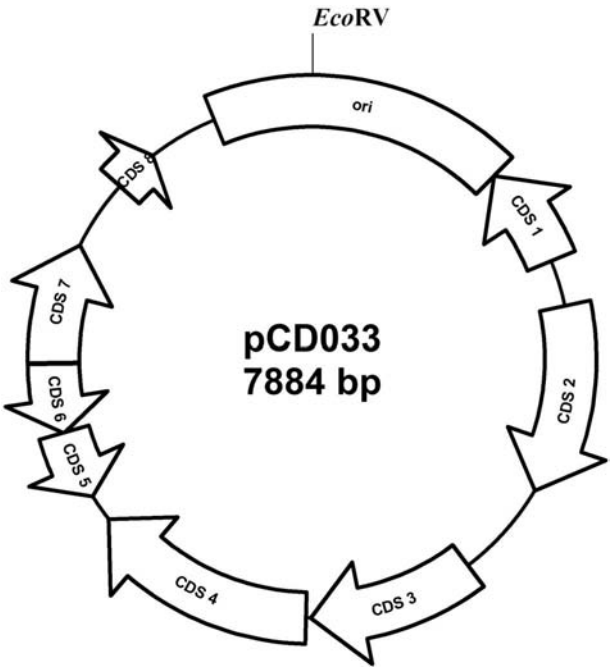


Fig. 1. Map of plasmid pCD033. Origin of replication and coding sequences are indicated. *Eco*RV cleavage site marks position 1.

dilutions from 10 ng samples, using SensiMix™ SYBR & Fluorescein Kit (Bioline) in a reaction volume of 30 μl. Separate reactions were performed for each template dilution. The cycling conditions for all reactions were 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Average cycle threshold (qCt) values were determined after regression adjustment of the baseline by the CFX manager software (BioRad). Relative standard curves were determined from the five serial dilutions and the slope of the relative standard curve was used for amplification efficiency (E) calculation following the equations described by Škulj and colleagues (Skulj et al., 2008). The PCN was calculated for all dilutions of each sample, then averaged and SD was calculated.

2.6. Plasmid curing for pCD033 of L. plantarum CD033

2.6.1. Construction of the curing plasmid

The *pemI* gene, its putative promoter and the putative origin of replication (ORI) from plasmid pCD033 were amplified by PCR from *L. plantarum* CD033 total DNA using the primers pemI_R_*Pst*I and pCD033_ori1_R_*Kas*I (Table 1). The 4 kb fragment was ligated to the CAT expression cassette under the control of its native promoter. The selection marker was amplified by PCR with the primers CAT_*Kas*I_F and CAT_*Kas*I_R (Table 1) from pCD194 in total DNA of *Bacillus subtilis* subsp. *subtilis* SB202 (DSM 4393). The resulting plasmid, pCD033_P_{pemI}_pemI_CAT, was transformed directly into electro-competent *L. plantarum* CD033.

Novobiocin sodium salt (N1628, Sigma-Aldrich) was diluted to 20 mg ml^{-1} in ddH_2O and sterile filtrated. Novobiocin as plain curing agent failed to lead to plasmid loss. Therefore, in order to facilitate plasmid curing, transformants with curing plasmid pCD033_P_{pemI}_pemI_CAT were generated and grown in the presence of novobiocin. Primary screenings were performed with 2.5, 5, 7.5 and 10 μg ml^{-1} novobiocin in MRS-CM for one week and incubation at elevated temperature of 40 °C. Due to severe growth impairment at these settings, we further tried 2.5 and 5 μg ml^{-1} novobiocin in MRS-CM at 30 °C, incubation for one week and subsequent plating on selective plating on MRS plates containing 5 μg ml^{-1} CM in serial dilutions of OD_{600} 0.4. After a constantly absorbable decline in cell growth after two days, we also tested for growth in MRS-CM with 0.25 μg ml^{-1} novobiocin for two days, followed by plating as described above directly after the drop in cell density. After 48 h of incubation at 30 °C, the received colonies were replica plated onto fresh MRS plates as well as selective MRS agar and screened via colony PCR for colonies of *L. plantarum* CD033 lacking the native plasmid. Respective clones were picked and transferred to MRS plates with and without selective pressure. A clone with just the heterologous plasmid confirmed to lack pCD033 was identified and further transferred to MRS-plates without selection pressure for losing the artificially introduced plasmid pCD033_P_{pemI}_pemI_CAT.

2.6.2. Confirmation of the plasmid cured strain by Southern blot analysis

Southern blot analysis was performed following a standard protocol (Sambrook and Russel, 2001). In brief, total DNA was extracted of each strain of interest with DNeasy Blood & Tissue Kit (Quiagen) according to the manufacturer's instructions for Gram-positive bacteria. DNA was then subjected to restriction digest with *Sac*I, *Kpn*I, *Nhe*I, *Sph*I for fragmentation and facilitated detection of the chromosomal gene *cbh* (encoding conjugated bile hydrolase), proving that genomic DNA is present. Plasmid DNA was linearized with *Eco*RV, which is a single cutter of plasmid pCD033 and linearizes within oripCD033. Total DNA concentration was determined, and 5 μg DNA per lane were separated on a 0.7% (*w*/*v*) agarose gel at 110 V for 1 h. After electrophoresis, the gel was equilibrated in denaturing blotting buffer (0.4 M NaOH, 1 M NaCl) directly followed by overnight capillary blotting (Southern, 1975). Subsequently, the Amersham Hybond N+ nylon membrane (GE Healthcare) was washed twice with 2× SSC and baked at 120 °C for 30 min. The blot was performed for each individual probe in one combined blotting procedure.

The two probes homologous to the *cbh* gene or the pCD033 origin of replication were amplified from *L. plantarum* CD033 total DNA with HybriPol DNA polymerase (Bioline) using the primers repB_probe_F, ori_pCD033_probe_R, cbh_probe_F and cbh_probe_R (Table 1) and DIG labeled dNTPs (Roche). Probes were separately boiled for 10 min at 95 °C, immediately placed on ice and diluted in 30 ml hybridization buffer (5× SSC, 0.1% [*w*/*v*] N-lauroylsarcosine, 1% [*w*/*v*] blocking reagent and 0.02% [*w*/*v*] SDS) to a final concentration of 40 ng μl^{-1}. Afterwards, membranes were separately washed with 2× SSC, 0.1% (*w*/*v*) SDS, followed by washing twofold with 0.5× SSC, 0.1% (*w*/*v*) SDS at 50 °C and a final washing step in washing buffer (1× maleic buffer: 100 mM maleate, 0.3% [*v*/*v*] Tween, 150 mM NaCl set to pH 7.5 with NaOH) at room temperature overnight. Hereafter, membranes were incubated in 30 ml 1× maleic buffer blocking buffer with 1% (*w*/*v*) blocking reagent (Roche) for 45 min, then blocking buffer was discarded and membranes were incubated with 1:5000 diluted primary antibody (11093274910; Roche) in fresh blocking buffer for 2 h. Blots were then washed three times with washing buffer, and incubated in 1.25 ml CDP-Star™ for 5 min in the dark and then chemiluminescent detection of alkaline phosphatase (11218603910; Roche) was performed. A fluorometric signal was achieved with NBT/BCIP detection system (Thermo Scientific).

Both probes led to precise bands at expected size (data not shown). In order to use the same membrane for both probes simultaneously, we applied a hot SDS stripping procedure according to manufacturer's instructions (GE Healthcare). Then, we hybridized with both probes at the same time.

2.7. Defining the minimal stable replicon of pCD033

In order to define the minimal stable replicon of pCD033, six deletion constructs were generated by fusing variously sized fragments (Fig. 2) of the putative replication region of pCD033 to a chloramphenicol acetyl transferase (CAT) expression cassette, amplified from plasmid pC194 (Horinouchi and Weisblum, 1982). Fragments were amplified from plasmid DNA by PCR using KOD DNA polymerase with primers listed in Table 1. The resulting plasmids, designated pCD033_I to VI were directly used to transform *L. plantarum* 3NSH by electroporation (Spath et al., 2012a). The segregational stability of the deletion constructs was determined by daily passaging the recombinant *L. plantarum* 3NSH clones in absence of antibiotic selection at 30 °C for more than 100 generations. Samples were taken every second day and 10^{-7} dilutions were replica plated on MRS plates with (10 μg ml^{-1} CM) and without

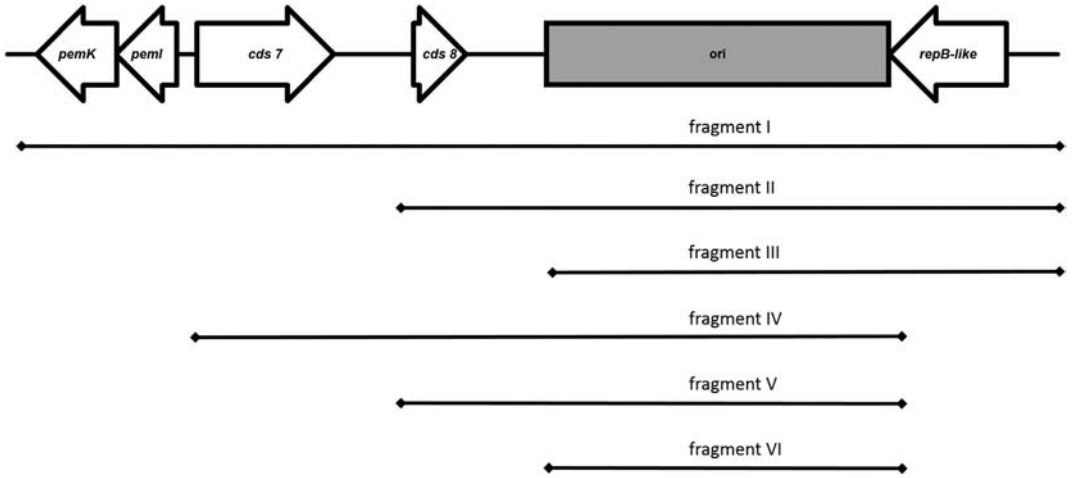


Fig. 2. pCD033 fragments (pCD033_I to VI) used for generation of the deletion constructs and plasmid stability analysis.

antibiotic selection. Determinations were performed in duplicates. Segregational stability was calculated by comparing the number of colonies present on selective and non-selective MRS plates.

3. Results and discussion

3.1. DNA sequence of pCD033, sequence analysis and prediction of putative coding sequences

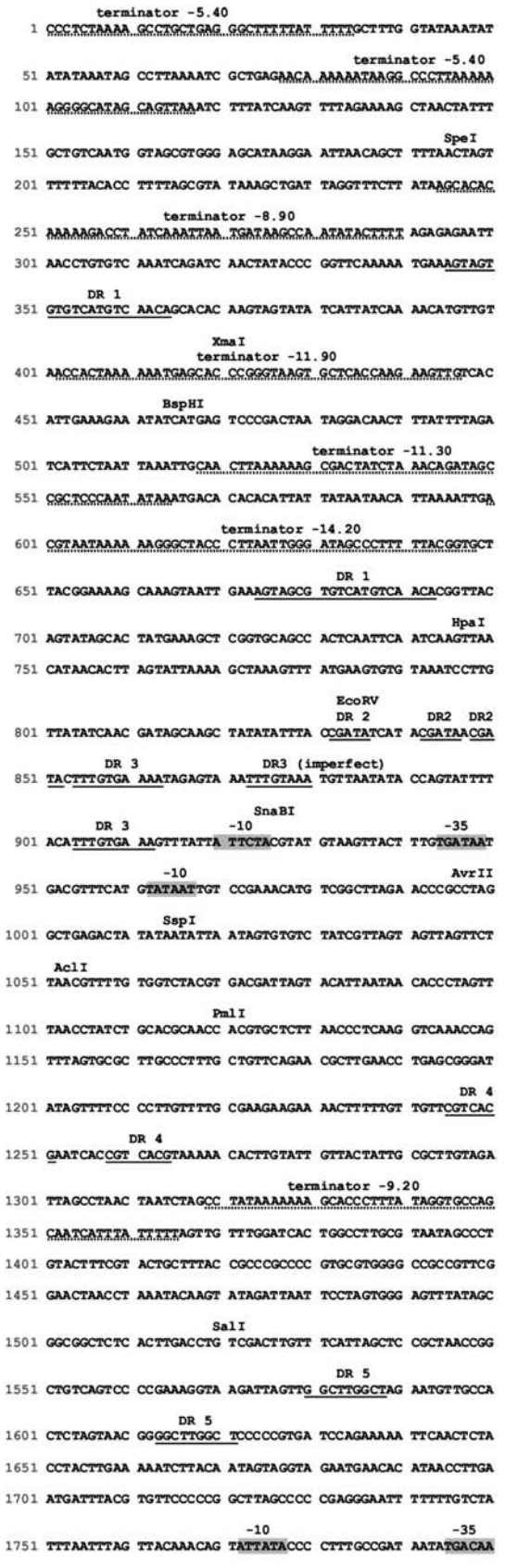
Agarose gel electrophoresis of isolated plasmid DNA from the silage strain *L. plantarum* CD033 indicated that the strain harbors a single plasmid. Sequence analysis confirmed a circular molecule. The plasmid designated pCD033, has a size of 7884 bp and a G + C content of 35.5%. In comparison, *L. plantarum* WCFS1 chromosomal DNA has a G + C content of 44.5% (Kleerebezem et al., 2003), suggesting that the plasmid was originally acquired by horizontal gene transfer. The plasmid map (Fig. 1) indicates the positions of putative CDSs and of the minimal stable origin of replication. The first nucleotide within a unique *Eco*RV site was arbitrarily designated as position 1. The sequence data of pCD033 CDSs have been deposited at the NCBI GenBank database and are available under accession number KP718939.1.

Open reading frames (ORFs) were predicted by using the pDRAW32 software. 14 ORFs were identified, whereof eight were inferred as coding sequences, due to the presence of a ribosomal binding site and/or similarity of the translated nucleotide sequence to already known proteins (Table 2). BLAST analysis revealed that CDS 1 encodes a protein of 167 amino acids (aa) sharing 99% identity with a hypothetical protein from *L. plantarum* G63 (Xi et al., 2013), 45% identity with a RepB protein from *Lactobacillus buchneri* plasmid pCD034-3 (Heinl et al., 2012) and 41% identity with an OrfX-like protein from *L. plantarum* (Sørvig et al., 2005). Those proteins are frequently found in theta replicating plasmids isolated from LAB and are supposed to play a role in plasmid stability, though they do not seem essential for plasmid replication (Benachour et al., 1997).

CDS 2 starts with TTG as start codon and encodes a protein of 309 aa, sharing 99% identity with the IS30-family transposase TraISLpl1 from *L. plantarum* FB335, which is also widely distributed among other LAB (Nicoloff and Bringel, 2003). The corresponding left (IRL) and right (IRR) terminal inverted repeats (5′-TGGTAGATTGTAAAATTAATCCGAA-3′) were identified up and downstream of CDS 2 (Nicoloff and Bringel, 2003). Mismatches between IRL and IRR are underlined. Another imperfect inverted repeat (IR) was identified directly upstream of CDS 2 (5′-AGCTGATCTTTTGTCTCC-3′; mismatches are underlined). The two sides of this IR enclose the ribosomal binding site of CDS 2 and may be involved in translational control of the IS30-like transposase. Between CDS 2 and CDS 3 we identified a sequence containing an inverted repeat (5′-ATG TAA AAA TTC CTT CCT TTT GAG AGA CGA TTT ATT CAT TAT ACG CTC TCT TAA AAG TTG TCT CAG GAA TCA GCT TAC ATC CAC AGT TT-3′), which seems to be conserved in many large LAB plasmids, as for example in pCD034-3 (Heinl et al., 2012). The translated CDS 3 shares only little similarity to other bacterial

Table 2
Putative genes encoded on pCD033, deduced gene products and proposed protein function.

Putative CDSs	Position	RBS	Product size (aa)	Amino acid identity (%)	Proposed function of gene product
CDS 1	1482–979	AGGAG	167	45	RepB-like
CDS 2	1708–2637	AGGAG	309	99	IS30-family transposase
CDS 3	3127–3948	AAGGA	273	–	Hypothetical protein
CDS 4	3970–5079	AAGAGG	369	36	Hypothetical protein, DGQHR-domain
CDS 5	5555–5211	AGAAGG	114	99	PemK-like, toxin
CDS 6	5812–5549	AGGAGG	87	100	PemI-like, antitoxin
CDS 7	5898–6485	GGAGG	280	97	Tyrosin recombinase
CDS 8	6840–7052	–	70	94	Conserved hypothetical protein


terminator -5.40
1 CCCTCTAAAA GCCTGCTGAG GGCTTTTTAT TTTTGCTTTG GTATAAATAT
terminator -5.40
51 ATATAAATAG CCTTAAAATC GCTGAGAACA AAAAATAAGG CCCTTAAAAA
101 AGGGGCATAG CAGTTAAATC TTTATCAAGT TTTAGAAAAG CTAACTATTT
SpeI
151 GCTGTCAATG GTAGCGTGGG AGCATAAGGA ATTAACAGCT TTTAACTAGT
201 TTTTTACACC TTTTAGCGTA TAAAGCTGAT TAGGTTTCTT ATAAGCACAC
terminator -8.90
251 AAAAAGACCT ATCAAATTAA TGATAAGCCA ATATACTTTT AGAGAGAATT
301 AACCTGTGTC AAATCAGATC AACTATACCC GGTTCAAAAA TGAAAGTAGT
DR 1
351 GTGTCATGTC AACAGCACAC AAGTAGTATA TCATTATCAA AACATGTTGT
XmaI
terminator -11.90
401 AACCACTAAA AAATGAGCAC CCGGGTAAGT GCTCACCAAG AAGTTGTCAC
BspHI
451 ATTGAAAGAA ATATCATGAG TCCCGACTAA TAGGACAACT TTATTTTAGA
terminator -11.30
501 TCATTCTAAT TAAATTGCAA CTTAAAAAAG CGACTATCTA AACAGATAGC
551 CGCTCCCAAT ATAAATGACA CACACATTAT TATAATAACA TTAAAATTGA
terminator -14.20
601 CGTAATAAAA AAGGGCTACC CTTAATTGGG ATAGCCCTTT TTACGGTGCT
DR 1
651 TACGGAAAAG CAAAGTAATT GAAAGTAGCG TGTCATGTCA ACACGGTTAC
HpaI
701 AGTATAGCAC TATGAAAGCT CGGTGCAGCC ACTCAATTCA ATCAAGTTAA
751 CATAACACTT AGTATTAAAA GCTAAAGTTT ATGAAGTGTG TAAATCCTTG
EcoRV
DR 2 DR2 DR2
801 TTATATCAAC GATAGCAAGC TATATATTTA CCGATATCAT ACGATAACGA
DR 3 DR3 (imperfect)
851 TACTTTGTGA AAATAGAGTA AATTTGTAAA TGTTAATATA CCAGTATTTT
SnaBI
DR 3 -10 -35
901 ACATTTGTGA AAGTTTATTA TTCTACGTAT GTAAGTTACT TTGTGATAAT
-10 AvrII
951 GACGTTTCAT GTATAATTGT CCGAAACATG TCGGCTTAGA ACCCGCCTAG
SspI
1001 GCTGAGACTA TATAATATTA ATAGTGTGTC TATCGTTAGT AGTTAGTTCT
AclI
1051 TAACGTTTTG TGGTCTACGT GACGATTAGT ACATTAATAA CACCCTAGTT
PmlI
1101 TAACCTATCT GCACGCAACC ACGTGCTCTT AACCCTCAAG GTCAAACCAG
1151 TTTAGTGCGC TTGCCCTTTG CTGTTCAGAA CGCTTGAACC TGAGCGGGAT
DR 4
1201 ATAGTTTTCC CCTTGTTTTG CGAAGAAGAA AACTTTTTGT TGTTCGTCAC
DR 4
1251 GAATCACCGT CACGTAAAAA CACTTGTATT GTTACTATTG CGCTTGTAGA
terminator -9.20
1301 TTAGCCTAAC TAATCTAGCC TATAAAAAAA GCACCCTTTA TAGGTGCCAG
1351 CAATCATTTA TTTTTAGTTG TTTGGATCAC TGGCCTTGCG TAATAGCCCT
1401 GTACTTTCGT ACTGCTTTAC CGCCCGCCCC GTGCGTGGGG CCGCCGTTCG
1451 GAACTAACCT AAATACAAGT ATAGATTAAT TCCTAGTGGG AGTTTATAGC
SalI
1501 GGCGGCTCTC ACTTGACCTG TCGACTTGTT TCATTAGCTC CGCTAACCGG
DR 5
1551 CTGTCAGTCC CCGAAAGGTA AGATTAGTTG GCTTGGCTAG AATGTTGCCA
DR 5
1601 CTCTAGTAAC GGGGCTTGGC TCCCCCGTGA TCCAGAAAAA TTCAACTCTA
1651 CCTACTTGAA AAATCTTACA ATAGTAGGTA GAATGAACAC ATAACCTTGA
1701 ATGATTTACG TGTTCCCCCG GCTTAGCCCC CGAGGGAATT TTTTTGTCTA
-10 -35
1751 TTTAATTTAG TTACAAACAG TATTATACCC CTTTGCCGAT AATATGACAA
1801 AGGGGTATTT

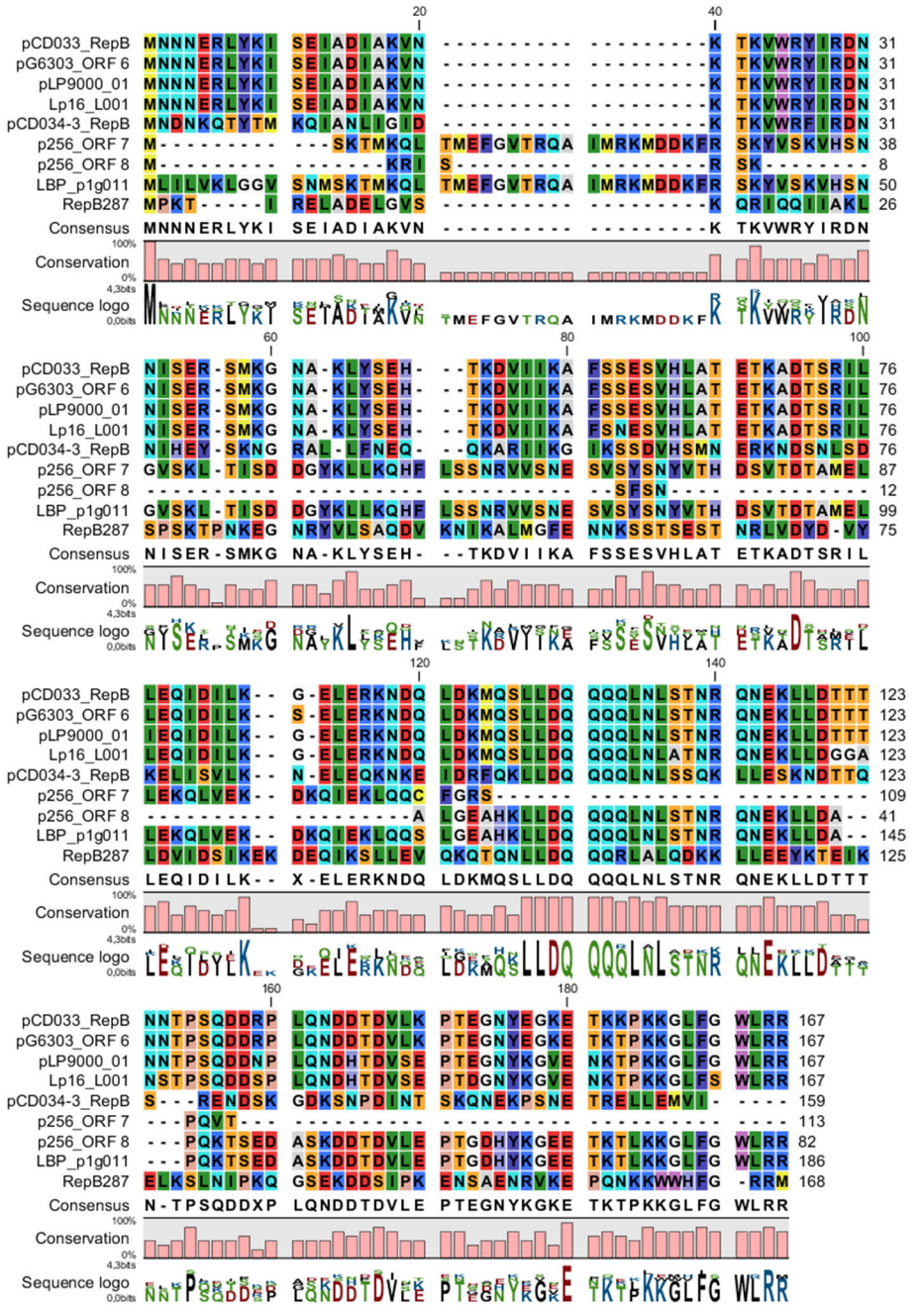
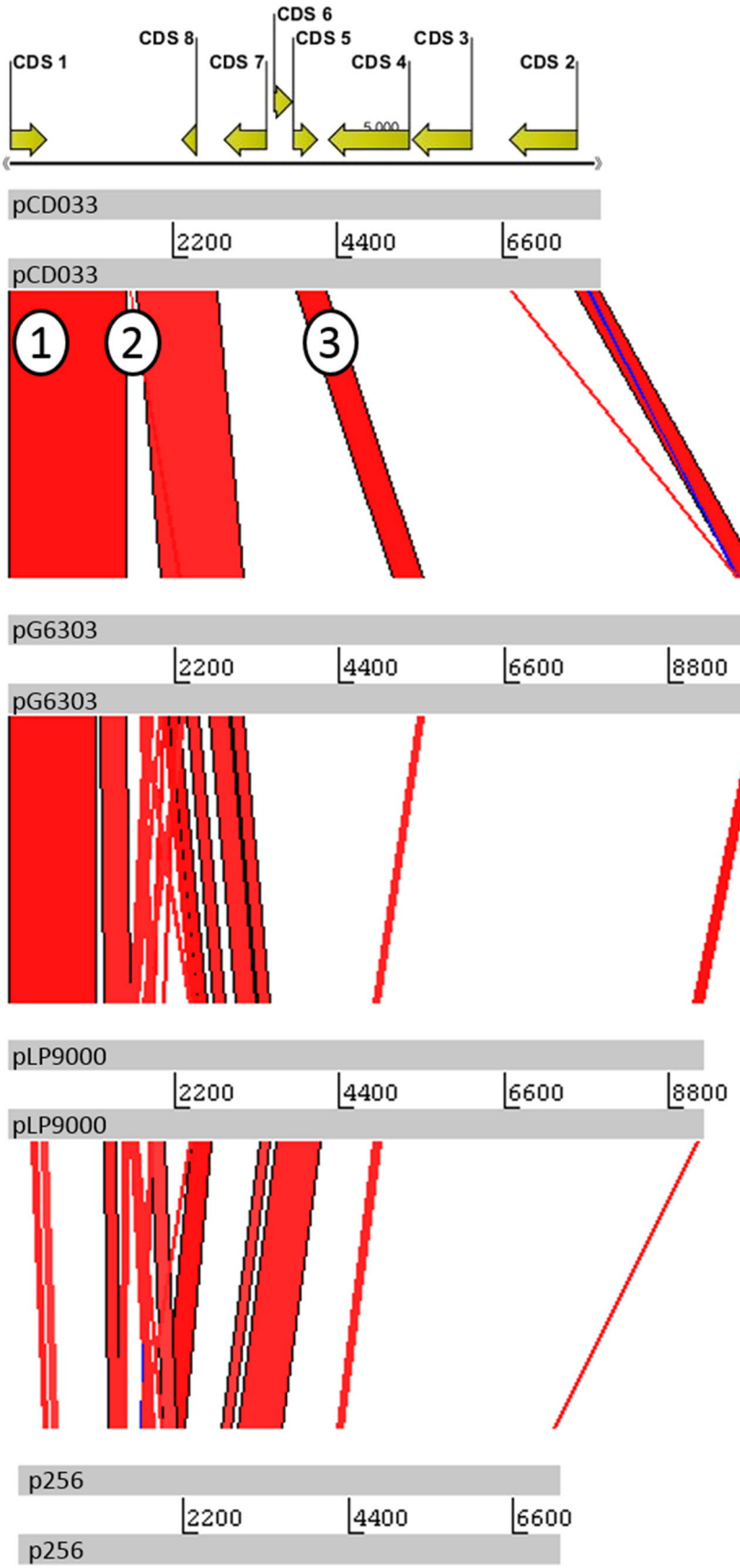


Fig. 4. Amino acid sequence alignment of the pCD033 CDS 1 gene product (RepB-like) with RepB/OrfX-like proteins from related plasmids pG6303, pLP9000, Lp16L, pCD034-3, p256, LBPp1, and pUCL287. In the case of p256, two consecutive ORFs (ORF 7 and ORF 8) were used for alignment. Dashes represent gaps introduced for optimal alignment. Respective amino acid positions are indicated on the right side. Similarities are indicated by conservation bars.

proteins so far. CDS 4 encodes a hypothetical DGQHR domain-containing protein of the DndB superfamily, sharing less than 40% similarity with a hypothetical protein from *Streptococcus sanguinis* and *Lactococcus lactis*. Some DGQHR domain proteins play a role in phosphorothioation of DNA (Liang et al., 2007) whereas others are, according to TIGRFAMs database entry TIGR03187, suggested to be involved in lateral gene transfer.

The proteins encoded by the overlapping CDSs 5 and 6 show 99% and 100% identity to *L. plantarum* subsp. *plantarum* P-8 PemK and PemI like proteins, presumably encoding a toxin–

Fig. 3. Replication region of pCD033. Direct repeats (DR) are underlined, putative-35 and -10 promotor regions are highlighted gray, and predicted terminators are indicated by a dotted underline. Free energies of stem-loop regions are given in kcal mol^{-1}. Plasmid position 1 in the *Eco*RV restriction site is indicated by an arrow.


CDS 1
CDS 8
CDS 7
CDS 6
CDS 5
CDS 4
CDS 3
CDS 2
5,000
pCD033
2200
4400
6600
pCD033
1
2
3
pG6303
2200
4400
6600
8800
pG6303
pLP9000
2200
4400
6600
8800
pLP9000
p256
2200
4400
6600
p256

antitoxin (TA) plasmid maintenance system. They seem to be translationally coupled and organized as an operon. Such plasmid addiction systems (PAS) have been described for Gram-negative as well as for Gram-positive bacteria (Sørvig et al., 2005; Siezen et al., 2012). It was reported for TA systems that they are highly abundant in free-living bacteria regardless of lifestyle and genome size, but TA systems are absent from the genomes of host-associated bacteria (Pandey and Gerdes, 2005). PAS were shown to serve as active support for stable maintenance of low copy-number plasmids, through neutralization of plasmid or chromosomally encoded toxin, by providing an antidote either based on protein or antisense RNA (Dmowski and Jagura-Burdzy, 2013). The principle of function of this system is that two proteins, a stable toxin and an unstable antitoxin, are antagonists. In case the plasmid is lost and the antitoxin subject to degradation, the more stable toxin remains intact within the cell and causes post-segregational killing. In *L. plantarum* NC7 plasmid p256 encodes a toxin–antitoxin-like system related to PemI and PemK from *E. coli* plasmid R1 (Sørvig et al., 2005). A PemI/PemK TA system was also reported to be present on an endogenous plasmid of *L. salivarius* (Fang et al., 2008). A *pemK* like gene was found on pG6303, one of the three cryptic plasmids of *L. plantarum* G63, but notably no cell toxin could be identified (Xi et al., 2013).

CDS 7 encodes an INT_C_like_3 protein which shows 97% and 96% identity, respectively, with putative tyrosine recombinases/integrases of *L. plantarum* (Malik et al., 2014) and *Pediococcus acidilactici*. The encoded protein belongs to a superfamily of DNA breaking/re-joining enzymes and might play a role in plasmid multimer resolution (Van Houdt et al., 2012).

CDS 8 encodes a protein highly conserved in versatile LAB. However, BLAST analysis failed to assign a specific function to the encoded hypothetical protein. Moreover, CDS 8 lacks an RBS, but a putative promoter region (-10 box 5′-TAACAAAAT-3′, -35 box 5′-ATGCAG-3′) was predicted upstream of CDS 8. The high degree of conservation of CDS8 among LAB species on nucleotide level leads us to the assumption that this sequence is part of a region that encodes regulative RNA rather than a protein.

A region on the plasmid (position 7053 to 978), lacking any ORFs larger than 35 codons, was supposed to play a role in plasmid replication of pCD033. Sequence analysis of this ORF-free region revealed AT-rich sequences, presence of putative transcriptional promoters and terminators, as well as direct (DR 1–5) and inverted repeats (Fig. 3). All these are essential elements of replication origins of bacterial replicons (Rajewska et al., 2012). On DNA-level, this region shows 97%, 97% and 89% identity (query coverage >85%), respectively, with the replication regions of *L. plantarum* plasmids pG6303, pLP9000, Lp16L (Daming et al., 2003; Sørvig et al., 2005; Crowley et al., 2013; Fan et al., 2015) and 94% identity (query coverage 83%) with the annotated genomic scaffold 00002 (GenBank: FR874849.1) from *L. pentosus* IG1 (Maldonado-Barragán et al., 2011). All these sequences share existence of a *repB*-like gene similar to that encoded on pCD033. They are thought to form a new family of plasmids regulated by an RNA-based replication mechanism, which has been described earlier for several plasmids (del Solar et al., 1998). Recently, an antisense RNA regulated replication has been proposed for *Staphylococcus areus* (Kwong and Firth, 2015) supporting our findings.

3.1.1. Comparison of ORF1 and pCD033 to related sequences

RepB and OrfX-like proteins of the previously described related plasmids pG6303 (Fan et al., 2015), pLP9000 (Daming et al., 2003), Lp16L (Crowley et al., 2013), pCD034-3 (Heinl et al., 2012), p256 (Sørvig et al., 2005), as well as LBPp1 (direct submission to NCBI data base, accession number NC_021233.1), and pUCL287 (Benachour et al., 1997) were compared on amino acid level, shown in Fig. 4. Similarities of pCD033 ORF1 gene-product (RepB-like) with RepB/OrfX-like proteins are indicated via conservation bars. In the case of p256, two consecutive ORFs (ORF 7 and ORF 8) were used for alignment (Sørvig et al., 2005). We hypothesize that ORF 7 and ORF 8 originally constitute one single open reading frame and were separated by an insertion of a single nucleotide (C at position 5377). BLAST analysis of the resulting protein shows highest similarities to LBP_p1g011, from plasmid LBPp1, without the frameshift observed in plasmid p256. Interestingly, we observe a strong conservation of 3 glutamine residues, preceded by Leu–Leu–Asp sequence (LLDQQQ) at the C-terminus, followed by a stretch of highly similar amino acids.

Plasmid pUCL287, which was reported to replicate via a novel theta replication mechanism, does not show significant homology to pCD033. RepB287 was shown to be involved in plasmid stability and regulation of plasmid copy number, but was not necessary for replication (Benachour et al., 1997). They reported that the N-terminus of RepB287 has high similarity to other lactococcal RepB and OrfX-like proteins, and has DNA-binding potential. Fig. 4 demonstrates homology to pCD033 ORF1 (RepB-like) in the C-terminus, including the conserved LLDQQQ-motif. Therefore, we assume a specific shared, yet unknown function of this C-terminal domain within the RepB/OrfX-like proteins.

Comparison of pCD033 with plasmids pG6303, pLP9000 and p256 is shown in Fig. 5. Pairwise sequence alignments starting with the RepB/OrfX-like protein translational start were performed using the Artemis Comparison Tool (ACT, http://www.webact.org/WebACT). Sequence homologies within the RepB/OrfX-coding region, the replication regions and the PemK coding region are indicated. Synteny is not high, but genetic elements such as toxin/antitoxin system were found in most related plasmids. Fig. 5 also indicates that relation of pCD033 is higher to pLP9000 than to p256. Interestingly, pLP9000 isolated from *L. plantarum* strain AS1.2986 was contrariwise shown to replicate via rolling circle replication (Daming et al., 2003).

Fig. 5. The Artemis Comparison Tool (ACT, http://www.webact.org/WebACT) was used for pairwise comparison of plasmid sequences of pCD033, pG6303, pLP9000, and pCD256. Nucleotide sequences were aligned from the RepB/OrfX-like protein translational start and visualized in ACT with a cut-off set to >22. Bars indicate regions of homology in the same orientation (red) and in inverted orientation (blue). Genetic organization of pCD033 is shown above its corresponding ruler. Plot reveals sequence homologies within the RepB/OrfX-coding region (1), the replication regions (2) and the PemK coding region (3).

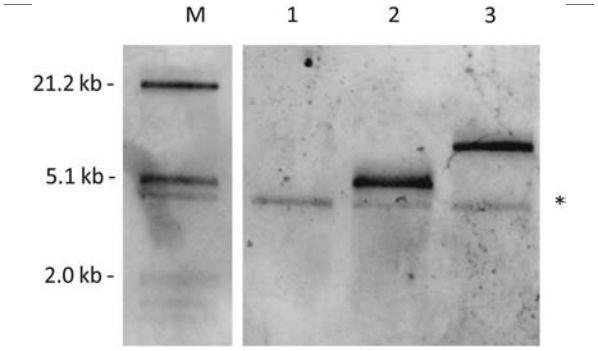


Fig. 6. Southern blot simultaneously hybridized with one chromosomal probe (cbh, indicated with an asterisk) and the plasmid (oripCD033). Lane M, DNA Molecular Weight Marker III; Lane 1, plasmid cured strain *L. plantarum* 3NSH; Lane 2, *L. plantarum* with heterologous plasmid pCD033_pemI_CAT (5 kb); and Lane 3, wild type strain *L. plantarum* CD033 with native plasmid (7.9 kb).

3.2. Plasmid copy number of pCD033

Total DNA of *L. plantarum* CD033 was extracted; two biological samples were measured in 5-fold dilutions and in triplicates. The chromosomal *cbh* gene (conjugated bile salt hydrolase) was used as a reference since only one copy is present per chromosome. By normalizing transcription of the target gene (on the plasmid) to that of the reference gene, we determined the relative plasmid copy number (PCN) of pCD033. The relative PCN was determined according to Skulj et al. (2008) and was calculated to be 2.9 ± 0.4 (PCN ± SD) copies per cell which means that pCD033 can be classified as a low copy number plasmid.

3.3. Plasmid curing

Several plasmid curing protocols are based on the addition of cell wall weakening substances such as novobiocin (Ruiz-Barba et al., 1991; Chin et al., 2005). Novobiocin is a well-known curing agent and has been described for plasmid curing in *Lactobacillus acidophilus* (Karthikeyan and Santosh, 2010), *L. plantarum* (Ruiz-Barba et al., 1991), *Lactobacillus salivarius* (Fang et al., 2008) and other *Lactobacillus* strains (Chin et al., 2005). However, in the case of *L. plantarum* CD033 the addition of novobiocin failed to result in plasmid loss. This might be due to the highly effective regulation of the toxin/antitoxin maintenance system present on pCD033.

Therefore, our strategy was to provide the antitoxin PemI by encoding the gene on a heterologous plasmid. Primarily, we tested transformation with heterologous plasmid pCD033_P_{pemI}_PemI_CAT, consisting of the pCD033 replication region, the *pemI* gene under control of its native promoter, and the CAT-expression cassette, in selective MRS-broth. According to the theory of competitive origins of replication, we assumed that just the presence of oriCD033 on pCD033_P_{pemI}_PemI_CAT would lead to competitive pressure on the native plasmid. Incompatibility of two identical origins of replication within one cell often leads to loss of one plasmid (Novick, 1987) and was previously described for *L. plantarum* (Bringel et al., 1989). Results however, showed that in this case the antibiotic pressure was not sufficient to displace the original plasmid. This could be assigned to different replication rates or other sequence specific features, favoring the native plasmids. Therefore, novobiocin was added to cells containing pCD033_P_{pemI}_PemI_CAT at various concentrations (2.5, 5, 7.5 and 10 μg ml^{-1}). Cells were cultivated for 7 days in combination

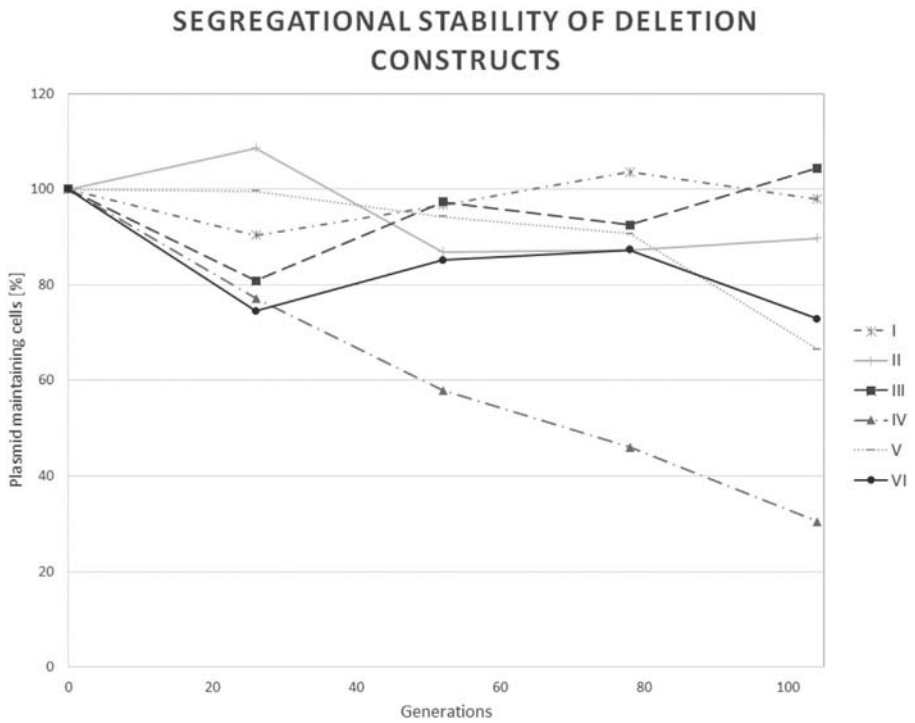


Fig. 7. Segregational stability of pCD033 deletion constructs. Fragments of pCD033 in deletion constructs I–VI are shown in Fig. 2. Values are means of duplicate determinations of plasmid carrying cells over a period of 100 generations.

with elevated temperature, in order to increase spontaneous loss of plasmid during segregation (Trevors, 1986). By using minimal concentrations of novobiocin (0.25 μg ml^{-1}) and two days of incubation at 30 °C we finally received desired clones of *L. plantarum* CD033.

Successful loss of the native as well as the heterologous plasmid pCD033_P_{pemI}_PemI_CAT was determined by colony PCR and Southern blot analyses (Fig. 6). Both probes were applied simultaneously on one blot after successful probing on two separate blots (data not shown). Additionally, we tested for eventual chromosomal integration by including extracted total DNA into the analysis. Three different strains, the plasmid free strain designated *L. plantarum* 3NSH (Fig. 6, lane 1), the intermediate strain lacking the native plasmid pCD033 still containing the heterologous plasmid (Fig. 6, lane 2) and the wild type strain *L. plantarum* CD033 (Fig. 6, lane 3) were subjected to Southern blot transfer. Fig. 6 shows simultaneous hybridization with the chromosome specific *cbh*-probe and a probe specific for the oripCD033, which is the origin of replication present on both plasmids. Thereby, we demonstrated that neither the native plasmid, nor the heterologous plasmid have integrated into the chromosome. Furthermore, 16rDNA was amplified and sequenced to confirm, that the plasmid free strain originated from *L. plantarum* CD033 and contaminations could be excluded.

Direct comparison of wild type strain *L. plantarum* CD033 with plasmid-cured strain *L. plantarum* 3NSH showed no significant difference in growth rates or recombinant protein expression (unpublished results). Thus, no essential or beneficial features seemed to be present on pCD033 under standard laboratory conditions, hence we cannot conclude that same applies for natural conditions (e.g. grass silage). The plasmid free strain was undistinguishable from the wild type strain in cultivation experiments.

3.4. Definition of the minimal stable replicon of pCD033

The minimal stable replicon of pCD033 was identified by transforming *L. plantarum* 3NSH with derivatives of the plasmid (Fig. 2) and testing segregational stability of the constructs under non-selective conditions. As indicated in Fig. 7, most constructs (pCD033_I, pCD033_II, pCD033_III, pCD033_V, pCD033_VI) are more or less stable for more than 100 generations in the strain *L. plantarum* 3NSH, which was cured form the original plasmid pCD033. Construct pCD033_IV lacks the *repB*-like gene and encodes the tyrosine recombinase function of CDS 7. This construct shows the lowest segregational stability after 100 generations with only 30% of the cells were still resistant to CM.

In summary, results indicate that CDS1 (high similarity to hypothetical LAB proteins and only 45% identity to RepB-like proteins) is not essential for plasmid replication. Deletion constructs lacking the *repB*-like gene yielded chloramphenicol resistant *L. plantarum* 3NSH (pCD033_V and VI), and same has been reported for p256 (Sørvig et al., 2005).

Interestingly, the putative tyrosine recombinase encoded by CDS 7 failed to increase segregational stability of construct pCD033_IV. Moreover, this construct with CDS7 (and without CDS1) turned out as the most instable construct. These findings were unexpected because tyrosine recombinases (like XerS from *Streptococci*) were shown to be involved in site-specific recombination and plasmid dimer/multimer resolution (Le Bourgeois et al., 2007; Johnston et al., 2015). Therefore, we expected improved proliferation of plasmid pCD033_IV to daughter cells, which however could not be observed (Fig. 7).

Summarizing, a section of the untranslated region of pCD033, flanked by CDS 1 (RepB-like protein) and its promoter region, was found to be the minimal stable replicon of pCD033 in *L. plantarum* 3NSH (in absence of antibiotic pressure). Contribution of the PemI/PemK plasmid maintenance system (pCD033_I) to plasmid stability could not be detected, since all plasmids, except pCD033_VI, remained stable over the whole investigation period.

4. Conclusions

Here we present sequencing, characterization and curing of the plasmid pCD033 from *L. plantarum* CD033, a generally regarded as safe (GRAS) strain isolated from stable grass silage. Plasmid pCD033 has a size of 7884 base pairs, contains 8 putative coding sequences and encodes a PemI/PemK-like toxin–antitoxin system. Moreover, plasmid pCD033 was shown to be maintained at a low plasmid copy number.

Furthermore, we describe an innovative curing strategy based on a curing vector consisting of the pCD033 replication region oripCD033 and the endogenous *pemI* antitoxin gene in combination with the curing agent novobiocin. The plasmid free strain was designated *L. plantarum* 3NSH and was used for analyzing plasmid stability of six different deletion derivatives of pCD033. Additionally, we illustrate a conserved C-terminal sequence of related RepB/OrfX-like proteins and pairwise alignment of pCD033 with related plasmids. Our data supports the idea of a novel, not yet elucidated putatively RNA-based, theta type replication mechanism of pCD033 which is possibly shared with related *L. plantarum* plasmids such as pG6303, pLP9000 and Lp16L (Daming et al., 2003; Crowley et al., 2013; Fan et al., 2015).

Acknowledgments

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Publication III

Evaluation of novel inducible promoter/ repressor systems for recombinant protein expression in *Lactobacillus plantarum*

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RESEARCH

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Evaluation of novel inducible promoter/repressor systems for recombinant protein expression in *Lactobacillus plantarum*

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Abstract

Background: Engineering lactic acid bacteria (LAB) is of growing importance for food and feed industry as well as for in vivo vaccination or the production of recombinant proteins in food grade organisms. Often, expression of a transgene is only desired at a certain time point or period, e.g. to minimize the metabolic burden for the host cell or to control the expression time span. For this purpose, inducible expression systems are preferred, though cost and availability of the inducing agent must be feasible. We selected the plasmid free strain *Lactobacillus plantarum* 3NSH for testing and characterization of novel inducible promoters/repressor systems. Their feasibility in recombinant protein production was evaluated. Expression of the reporter protein mCherry was monitored with the BioLector® micro-fermentation system.

Results: Reporter gene mCherry expression was compared under the control of different promoter/repressor systems: P_{lacA} (an endogenous promoter/repressor system derived from *L. plantarum* 3NSH), P_{xylA} (a promoter/repressor system derived from *Bacillus megaterium* DSMZ 319) and $P_{lacSynth}$ (synthetic promoter and codon-optimized repressor gene based on the *Escherichia coli lac* operon). We observed that P_{lacA} was inducible solely by lactose, but not by non-metabolizable allolactose analoga. P_{xylA} was inducible by xylose, yet showed basal expression under non-induced conditions. Growth on galactose (as compared to exponential growth phase on glucose) reduced basal mCherry expression at non-induced conditions. $P_{lacSynth}$ was inducible with TMG (methyl β-D-thiogalactopyranoside) and IPTG (isopropyl β-D-1-thiogalactopyranoside), but also showed basal expression without inducer. The promoter $P_{lacSynth}$ was used for establishment of a dual plasmid expression system, based on T7 RNA polymerase driven expression in *L. plantarum*. Comparative Western blot supported BioLector® micro-fermentation measurements. Conclusively, overall expression levels were moderate (compared to a constitutive promoter).

Conclusions: We evaluated different inducible promoters, as well as an orthologous expression system, for controlled gene expression in *L. plantarum*. Furthermore, here we provide proof of concept for a T7 RNA polymerase based expression system for *L. plantarum*. Thereby we expanded the molecular toolbox for an industrial relevant and generally regarded as safe (GRAS) strain.

Keywords: *L. plantarum* 3NSH, BioLector® micro-fermentation system, Orthologous expression system, T7 RNA polymerase, IPTG, Inducible expression

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Background

Lactobacillus plantarum is a versatile lactic acid bacterium that is generally regarded as safe (GRAS). It inhabits diverse ecological niches and exhibits probiotic characteristics [42]. *L. plantarum* is often used as starter or adjunct culture in fermented food and feed production processes like for sausages, cheeses, fermented vegetables, and grass or corn silage [10, 36, 37]. Due to its high oxygen tolerance and robustness in natural fermentation processes, *L. plantarum* has gained increasing interest also as a host for recombinant protein expression and thus, its use in biotechnological applications is steadily growing [1, 20, 41]. Research involves genomics, transcriptomics, cell engineering and evolutionary strain optimization [9, 37] e.g. for bulk production of chemicals, metabolites and enzymes [23, 28] as well as for in situ delivery of vaccines [8, 11, 12, 32, 50]. Anti-microbial features, such as plantaricin production, are also of growing importance [33].

Specific gene regulatory elements like promoters are a prerequisite for efficient transcription of recombinant genes in any host organism. Accordingly, several constitutive promoters and shuttle vector systems have been established [38, 43, 44, 47]. Often, constitutive expression is preferred, for example for in situ delivery of recombinant proteins in the human body, or when steady-state gene expression is required [38]. Contrarily, constitutive promoters do not allow regulation of gene expression and production levels are directly linked to cellular growth. Continuous transcription throughout the fermentation process poses a limit to the expression of foreign proteins, which are potentially toxic to the host cell or exhibit excessive metabolic burden.

An alternative strategy is to use substrate dependent promoters that can be induced after a certain cell density has been reached. Several inducible promoters for *L. plantarum* have been described in the literature. The nisin-controlled gene expression (*NICE*) system is inducible with the bacteriocin nisin from *Lactococcus lactis* and was established also for *L. plantarum* [25]. However, the expression is not tightly regulated except if the target expression cassette is integrated into the host's chromosome [34]. The pSIP system comprises a well-established inducible promoter system and is based on the induction of promoters from *Lactobacillus sakei* with an inducing peptide [46]. More recently, another inducible promoter based on manganese starvation was described for *L. plantarum* NC8 [3].

Yet, numerous other substrate induced promoter-repressor systems are present in LAB and other bacteria that eventually may serve to efficiently control transgene expression. *Lactobacillus plantarum* contains a *lac* -operon which was expected to be regulated similarly as the well-studied *lac*-operon of *Escherichia coli*, where the *lac*-operon comprises the genes *lacZ* (β-galactosidase), *lacY* (lactose permease), *lacA* (transacetylase) and *lacI* (repressor). Allolactose is the natural inducer of the *lac*-operon. In *E. coli*, thio-galactosides such as IPTG (isopropyl β-D-1-thiogalactopyranoside) and TMG (methyl β-D-thiogalactopyranoside) are the most commonly used inducers in industrial production processes.

We established a synthetic inducible promoter system based on the *E. coli* derived *lac*-operon, which we adapted for *L. plantarum* in the high copy number shuttle vector pCDLbu1 [15, 43]. Based on the inducible synthetic system, we designed and constructed an artificial T7 RNA polymerase regulated dual plasmid expression system and demonstrated its applicability in *L. plantarum* 3NSH. Additionally, we tested endogenous *lac*-operon regulatory sequences from *L. plantarum* 3NSH. This strain is derived from *L. plantarum* CD033, which was cured of its native plasmid [17]. Plasmid free strains are preferable expression hosts, since native plasmids sometimes interfere with expression vector replication.

Another well-known regulated system is the xylose operon and the xylose promoter/repressor gene from *Bacillus megaterium*, which is well established for Gram-positive bacteria, and was already used for high yield production of secretory levansucrase in *B. megaterium* YYBm1 [22] and dextransucrase in *B. megaterium* MS941 [26]. Moreover, three different recombinant proteins in *Brevibacillus choshinensis* SP3 under the control of P_{xylA} from *B. megaterium* have been reported [5]. D-xylose is metabolized by two intracellular enzymes: the D-xylose isomerase (XylA) and the D-xylose kinase (XylB). D-xylose can be transported into the cell by two different mechanisms. One mechanism involves a D-xylose-H^+ or $-Na^+$ symporter (*xylT*) and is regulated by CcpA [40]. Another mechanism is driven by ATP and consists of a high-affinity xylose transporter system involving a periplasmic binding protein. For three species of facultative hetero-fermentative lactobacilli, *Lactobacillus pentosus*, *L. plantarum*, and *Lactobacillus casei* it was shown that EII^{Man} complex of the phosphoenolpyruvate (PEP): D-mannose phosphotransferase system (PTS) is involved in D-xylose transport via facilitated diffusion [4]. Posno and co-workers [35] reported that *L. plantarum* does not metabolize D-xylose. For its use as an inducer, this is an advantage, since D-xylose is not degraded and keeps the level of induction constant throughout the process.

In this study, we present the establishment and characterization of different inducible promoter/repressor systems (and their respective inducer) in the high copy number pCDLbu1 shuttle vector for *L. plantarum* 3NSH. We used mCherry as reporter protein and expression

levels were analyzed with the BioLector® micro-fermentation system and confirmed by Western blot immunodetection. Furthermore, we established an inducible T7 RNA polymerase based system for regulated recombinant gene expression. Summarizing, we present expression plasmids with a set of novel inducible promoters, and expand the toolbox for recombinant protein expression in *L. plantarum*.

Results and discussion

Different inducible promoter systems were characterized and tested in the plasmid free strain *L. plantarum* 3NSH [17]. Comparative studies were carried out regarding bacterial growth rates, level of reporter gene expression, effect of inducer and behavioral differences due to varying carbon sources. BioLector® micro-fermentations were established. FlowerPlates (with or without optodes for low pH and dissolved oxygen) for detection of biomass (calculated optical density) and fluorescence were used for *L. plantarum* fermentation and analysis.

Besides promoter elements and transcription initiation, other factors additionally influence the level of protein expression. Such are terminators, untranslated regions, plasmid copy numbers and the protein itself (amino acids, folding, and toxicity). In our experimental set-up, we chose the ribosomal binding site (RBS) and the spacer between the RBS and start codon to be identical for all promoter constructs (Fig. 1), in order to exclude

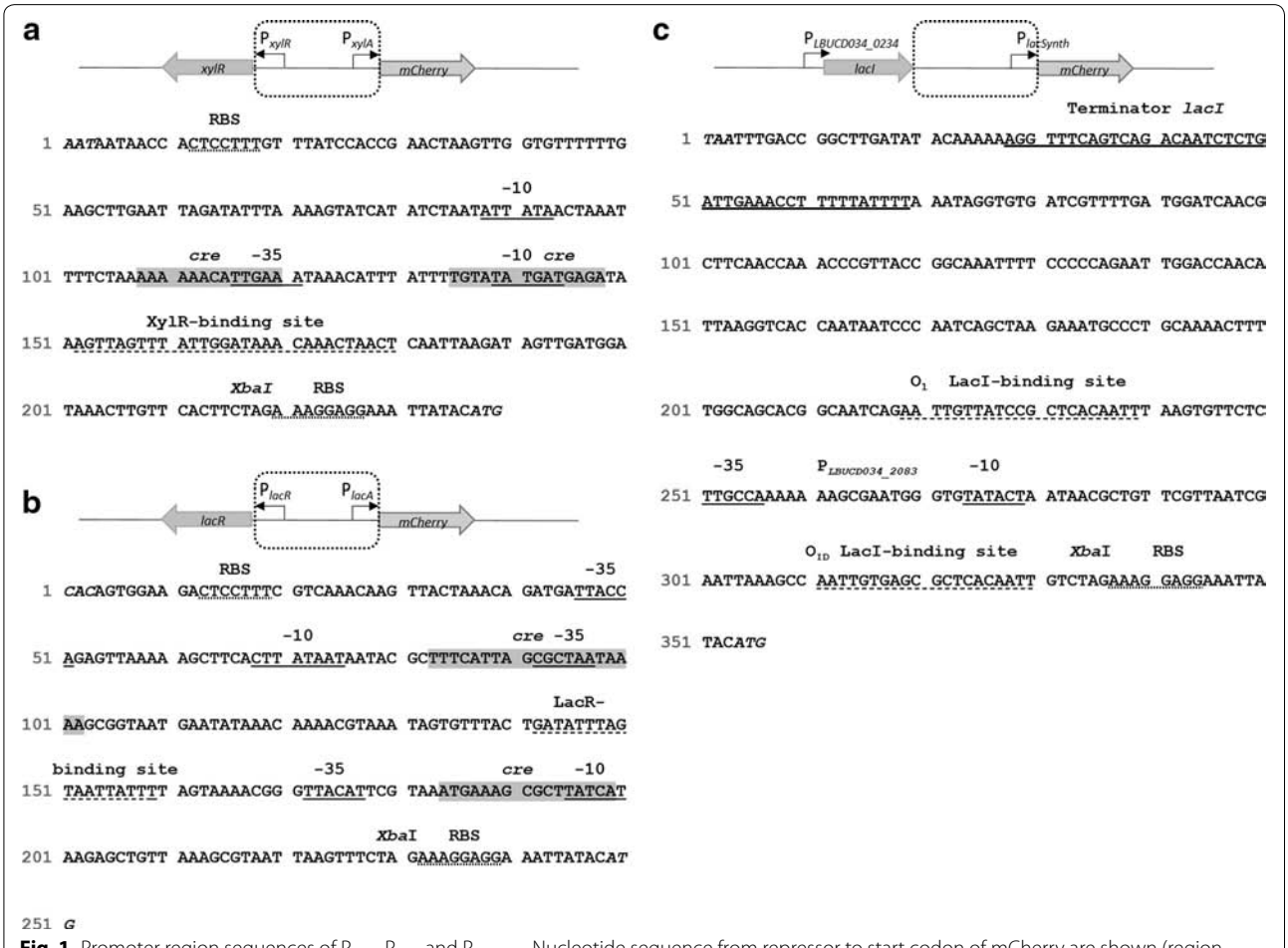

Fig. 1 Promoter region sequences of P_{xylA}, P_{lacA} and $P_{lacSynth}$. Nucleotide sequence from repressor to start codon of mCherry are shown (region within *dotted square*). The mCherry start codon is indicated in *italics*, preceded by an identical ribosome binding site (*RBS; italics*) an *Xba*I restriction site (*bold*) and an identical 9 nt spacer sequence was introduced upstream of mCherry start codon. The −35 and −10 promoter region were identified (SoftBerry, BPROM) and are *underlined*. Primer binding sites for negative controls (for construction of negative controls without promoter) are underlined in *dashed line*. **a** P_{xylA}; promoter of *xylA* gene from *B. megaterium* DSMZ 319 and promoter of repressor XylR. Operator sequences for XylR binding are underlined; *cre* sites (catabolite-responsive element) are *highlighted*. **b** P_{lacA}; endogenous promoter of LacA from *L. plantarum* 3NSH and promoter of repressor LacR. LacR-binding site was identified (RegPrecise) and *underlined*, and putative *cre*-sites are highlighted. **c** $P_{lacSynth}$; promoter P_{2083} from *L. buchneri* CD034 with artificially integrated operator binding sites with recommended distance of 93 nt (O_1 and O_{ID} from *E. coli*) are *underlined* (*dotted line*), terminator of *lacI* is *underlined* (*solid line*)

any translational effects on mCherry expression caused by different RBSs. However, the promoter consensus sequences (including the −35 and −10 region) were specific for each tested promoter.

The heterologous promoter P_{xylA} is inducible by xylose

The promoter fragment P_{xylA} and the repressor gene cassette XylR were amplified from *B. megaterium* DSMZ 319 genomic DNA with primers listed in Table 1. Nucleotide sequences of promoters P_{xylA} and promoter P_{xylR} to start codon of mCherry are shown in Fig. 1a, where promoter, RBS, *cre* (catabolite-responsive element)-sites and XylR binding site are indicated. The xyl-repressor binding motif is indicated according to Stammen and colleagues [45].

Description of the operon and its regulation was presented by Schmiedel and colleagues [40]. Preliminary tests were performed with native RBS from XylA from *B. megaterium* DSMZ 319 in pCDLbu1_$P_{xylA(nativeRBS)}$–mCherry. The comparison of RBS and spacer sequence of $P_{xylA\ (native\ RBS)}$ and P_{xylA} is shown in Additional file 1: Figure S1. We compared mCherry expression with native RBS to the uniform and artificial SOPT#9 spacer RBS and sequence (5′-*TCTAGA*AAGGAGGAAATTATA-**CATG**-3′, from *Xba*I to start codon), which was established for *L. plantarum* CD033 [47]. SOPT#9 was used for all constructs and allowed comparison of mCherry expression apart from translational influences. Interestingly, we found that SOPT#9 lead to slightly higher expression levels compared to the native *xyl*A RBS and

Table 1 List of primers used in this study

Name	5′–3′ Sequence
PlacSynth_*Sac*I_*Eco*RI_F	GATGAC**GAGCTC***GAATTC*TGGTCTTTATTCTTCAA
M13_R_*Nhe*I	CGACGA**GCTAGC**AGCCAGGAAACAGCTATGACC
mCherry_RBS_*Xba*I	GCTGCT**TCTAGA**AAGGAGGAAATTATACATGTTATCAAAGGGTGAAGAAG
mCherry_R_*Bam*HI	CGTCGT**GGATCC**TTATCACTTGTATAATTCATCCATACC
Tldh_amp_R_*Pst*I	CTGCTG**CTGCAG**AAAAAGATTAAAAAAGCCGCTGC
mCherry_seq_R	TGGACGACCTTCACCTTCAC
mCherry_seq_F	AACGTATGTACCCAGAAGATG
CAT_seq2_back	TACATCATTCTGTTTGTGATGG
B_mega_XylOP_out_F	AACATATAAACAGCCAGTTGCC
B_mega_XylOP_R(*Spe*I, *Sca*I, *Bam*HI)	GTAGTA**GGATCC***AGT***ACTAGT**TTCCCCCTTTGATTTAAGTG
mCherry_w/o_RBS_*Xba*I	CGTCGT**TCTAGA**ATGTTATCAAAGGGTGAAGAAGATAAC
p256_miniori_for	CATCATAAGCTTCCCGCACGCATAGCGGTGC
B_mega_XylOP_F_*Mfe*I, *Kpn*I	GTAGTA**CAATTG***GGTACC*AAGGTGAGGGTGGAGACAG
Bmega_XylR_newRBS_*Xba*I_Phos_R	GTATAATTTCCTCCTT**TCTAGA**AGTGAACAAGTTTATCCAT
mCherry_Phos_F	ATGTTATCAAAGGGTGAAGAAG
B_mega_XylOP_seq_F	CAATTCCGATATTAATACTGATG
B_mega_XylOP_seq_R	CTAGTCGGAATAGGAATTTGTG
LacI_Lplant_F_*Sac*I	AGCAGC**GAGCTC**CCTAATAGAACTGCGGTGGTC
LacI_Lplant_R_*Xba*I	AGCAGC**TCTAGA**AACTTAATTACGCTTTAACAGC
lacR_Gal_seq_R	AATTGAAGTGATGCGGGTCTG
lacR_Gal_seq_F	AATTGCGCCAGCTAACACCC
T7_RNAP_Lp_RBS	CAGCAGTCTAGATCCTAAAGGAGG
T7_RNAP_Lp_Term_R_*Sal*I	CAGCAG**GTCGAC**TTGATATACAAAAAAGG
M13_2_F	TTGTAAAACGACGGCCAGTG
T7-Promoter_*Sac*I	GCTGCT**GAGCTC**AGATCGATCTCGATCCCGCG
T7-Terminator_*Sal*I	GCTGCT**GTCGAC**TCCGGATATAGTTCCTCCTTTC
ery_back_*Kas*I	CATCAT**GGCGCC**TCCGATTGCAGTATAAATTTAACG
oripE194_seq_back	AATCAAATCGGTATAAATCTGAC
Ery_F_*Nhe*I	CATCAT**GCTAGC**TCCGATTGCAGTATAAATTTAACG
Pempty_*Sac*I_R	TAGTAG**TCTA***GAGCTC*GAATTCACTGGCCGTCG
mCherry_RBS_*Sac*I_F	GCTGCTGAGCTCAAGGAGGAAATTATACATGTTATCAAAGGGTGAAGAAG

Restriction sites are underlined and highlighted in bold or italics

spacer sequence (data not shown) and was well suited for recombinant protein expression in *L. plantarum* 3NSH. Parental rolling circle replicating plasmid pCDLbu1 is shown in Fig. 2a. The final shuttle vector pCDLbu1_P_{xylA}_mCherry for P_{xylA} regulated mCherry expression is depicted in Fig. 2b.

Cells were grown on selective media with either glucose (Fig. 3a, c) or galactose (Fig. 3b, d) as main carbon source, induced with xylose (or absence of inducer) after 2 h and analyzed. Figure 3a, b show relative fluorescence units (RFUs) of mCherry expression (with or without induction) under control of P_{xylA} for 23 h. In related literature the used amount of xylose added as inducer varies from 0.5 % (*w*/*v*) in *Bacillus megaterium* to 0.2 and 2 % in *B. subtilis* [2, 22, 26]. Figure 3a shows that the addition of 0.2 or 2 % xylose in MRS medium with glucose as main carbon source showed no effect on mCherry expression as compared to non-induced conditions. Figure 3b indicates that growth on galactose and induction with 0.2 or 2 % xylose led to enhanced expression of mCherry expression during exponential growth phase. Moreover, basal expression in medium containing 2 % galactose as main carbon source was repressed during the exponential phase (Fig. 3b), as compared to growth on glucose (Fig. 3a). *Lactobacillus plantarum* 3NSH is incapable of metabolizing xylose (data not shown), but xylose is efficiently transported into the cell. The use of this promoter/repressor-system in lactobacilli was tested here for the first time.

Additionally, we tested a negative control (expression plasmid without the promoter/repressor fragment), which did not show any mCherry expression (Fig. 3a, b, solid line). Thus, basal expression was caused by weak repression of P_{xylA} through inefficient XylR repressor binding and not by any putative additional regulatory sequences present on the plasmid.

In *B. megaterium*, the presence of glucose was shown to cause repression of P_{xylA} by CcpA (catabolite control protein A) binding *cre*-sites within the promoter region and the *xylA* gene [13]. The *xylA* promoter in our context (Fig. 1a) contains two *cre*-sites, which were termed *cre**-35.5 and (*cre*)-8.5 (a *cre*-like site) by Gösseringer and coworkers [13] who also showed that in *B. megaterium* the *cre* + 130.5 (within *xylA* sequence) and *cre**-35.5 are simultaneously bound by CcpA, which results in looping of intervening DNA and tight repression of *xylA* transcription. Interestingly, we did not observe catabolite repression of mCherry expression by the presence of glucose with our construct (Fig. 3a). A reason for

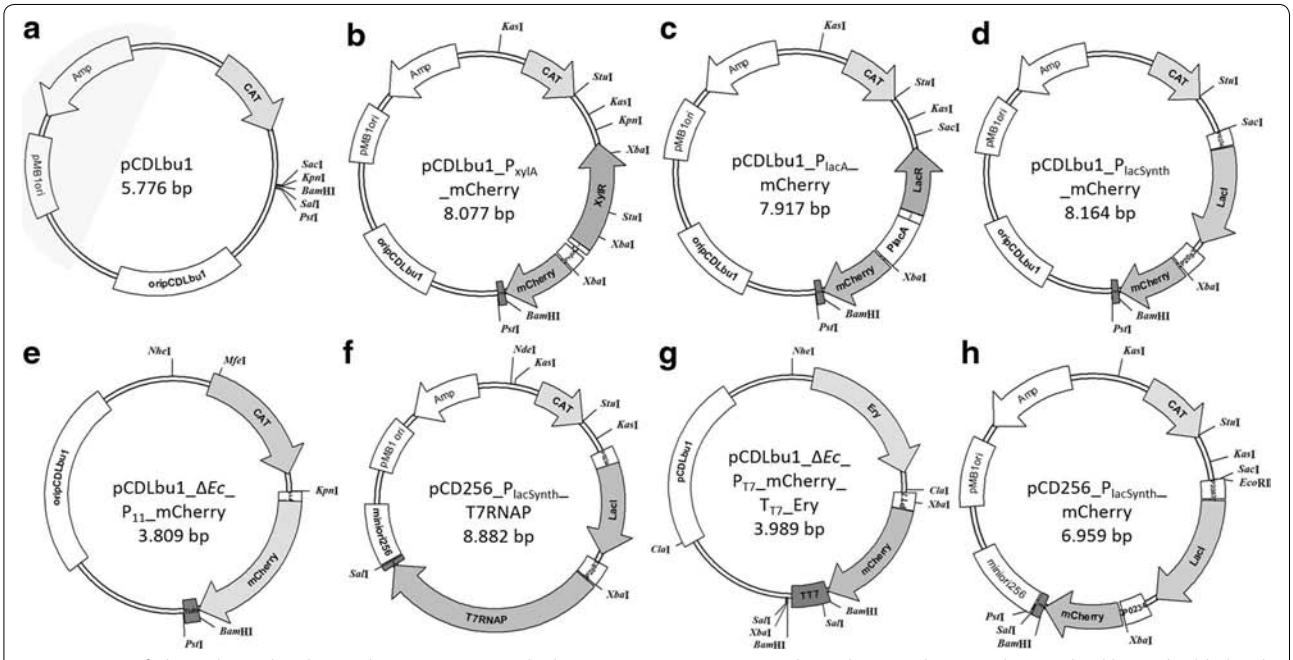

Fig. 2 Maps of plasmids used in this study. Annotations and relevant restriction sites are indicated. **a** pCDLbu1 initial vector backbone (highlighted region are *E. coli* specific sequences); **b** pCDblu1_P_{xylA}_mCherry; **c** pCDLbu1_P_{lacA}_mCherry; **d** pCDLbu1_$P_{lacSynth}$_mCherry; **e** pCDLbu1Δ*Ec*_P_{11}_mCherry (constitutive P_{11} promoter; internal reference plasmid described by Tauer and colleagues [47]); The term '*ΔEc*' indicates removal of *E. coli* specific sequences which are highlighted in plasmid A. **f** pCD256_$P_{lacSynth}$_mCherry; **g** pCD256_$P_{lacSynth}$_T7RNAP; **h** pCDLbu1Δ*Ec*_P_{T7}_mCherry _T_{T7}_Ery. OripCDLbu1 and miniori256: origins of replication (ori) for *L. plantarum* 3NSH; pMB1ori: ori for replication in *E. coli*; *CAT* chloramphenicol acetyltransferase gene; *Amp* ampicillin resistance gene; *Ery ermI* gene encoding resistance to erythromycin; *P* promoter; *T* terminator of transcription. Subscripted characters are specifications. Important restriction sites are indicated

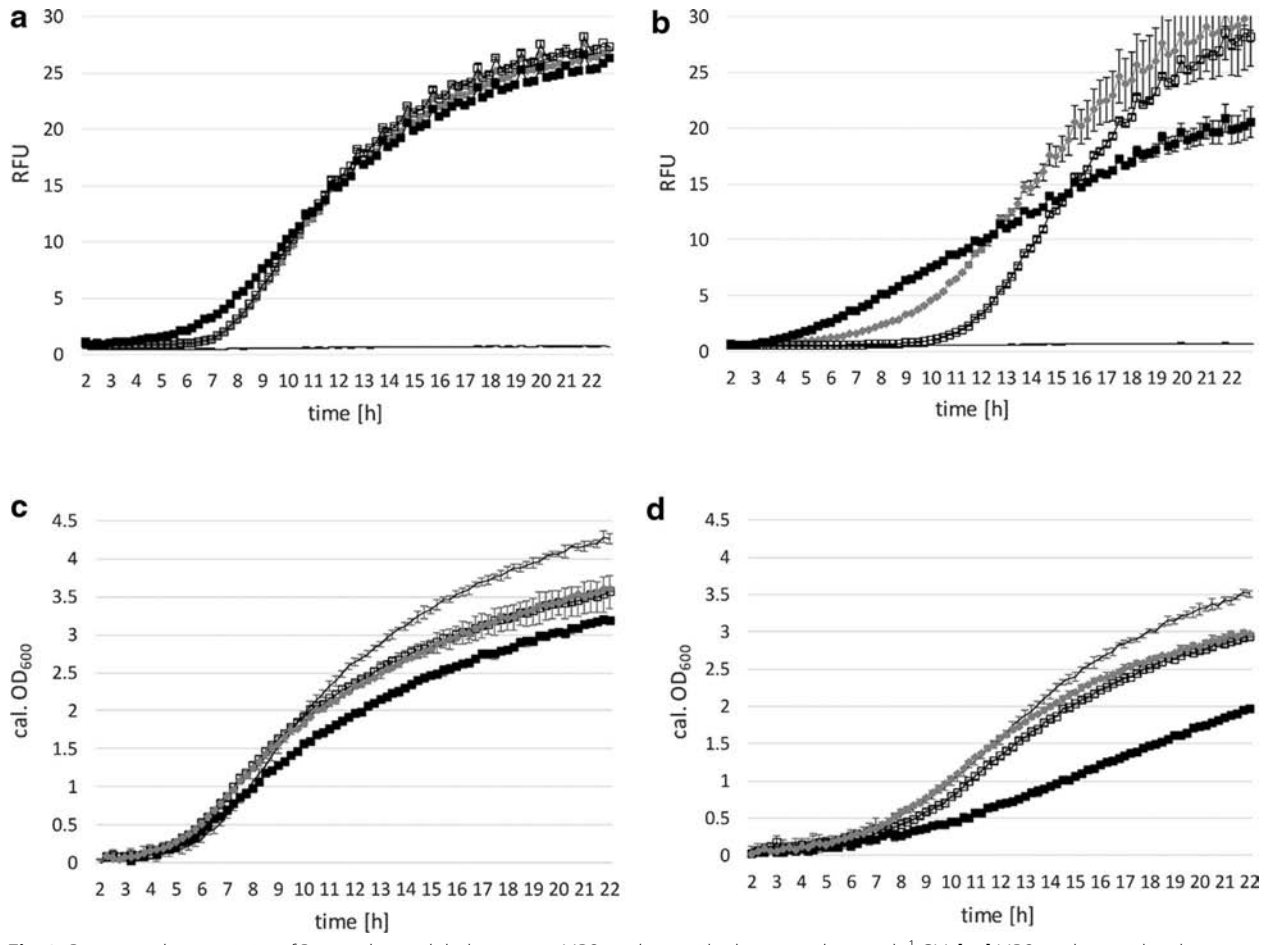

Fig. 3 Promoter characteristics of P_{xylA} and growth behavior. **a**, **c** MRS medium with glucose and 5 µg ml^{-1} CM; **b**, **d** MRS medium with galactose and 5 µg ml^{-1} CM, mean values of four replicates are given and standard deviations are indicated. *Filled square* induced 2 % xylose. *Filled diamond* induced 0.2 % xylose. *Square* non-induced. *Solid line* negative control. **a**,**b** specific expression levels of mCherry under control of P_{xylA} after induction with 2, 0.2 % xylose (or no induction) after 2 h in BioLector® micro-fermentation. Change of mCherry expression (RFUs, relative fluorescent units) over time (hours) of P_{xylA} mediated expression in comparison to the negative control is shown. **c** and **d** corresponding calculated OD_{600} values

this could be the lack of *cre* + 130.5 within the mCherry gene, hence, multimer formation and efficient catabolite repression is prevented. Another explanation for the relatively strong basal expression level could be that glucose inhibits DNA binding by XylR and acts as a low-efficiency inducer for XylR as reported by Dahl and co-workers [6]: similar structure of xylose and glucose enable both sugars to utilize the same binding site on repressor XylR.

Plasmid pCDLbu1_P_{xylA}_mCherry containing cells only showed minor growth differences on selective medium with either glucose or galactose (Fig. 3c, d). Growth on galactose slightly increased mCherry expression and decreased basal expression levels, resulting in an improved regulation of the system during exponential phase (Fig. 3b). We hypothesize that galactose interferes less with XylR mediated repression in *L. plantarum* than glucose and, hence, leads to improved repression of mCherry expression.

Lactobacillus plantarum 3NSH does not metabolize xylose, but effective transportation of xylose was demonstrated through inducibility of expression. The *L. plantarum* WCFS1 complete genome sequence [21] suggests genes involved in transport (lp_0331, lp_0975), but no *xylA* or *xylB*. Chaillou et al. [4] report that EII^{Man} complex of the phosphoenolpyruvate (PEP): D-mannose PTS is involved in D-xylose transport via facilitated diffusion. For industrial processes, it is considered an advantage, when the inducing substance is not degraded and a constant concentration during cultivation can be maintained. In terms of plant based biomass degradation, where xylose is highly abundant, this expression regime could provide a self-inducing promoter system for the

production of e.g. endoglucanases and xylanases, thereby increasing the rate and efficacy of substrate metabolism in ensiling processes.

The endogenous promoter P_{lacA} is inducible by lactose

The promoter of *lacA* (β-galactosidase) and the promoter of the Lac repressor (*lacR*) were amplified from *L. plantarum* 3NSH genomic DNA with primers shown in Table 1. Figure 1b shows the nucleotide sequences of endogenous promoters P_{lacA} and promoter P_{lacR} in divergent orientation. LacR binding site, *cre*-site and RBS are indicated. The final shuttle vector pCDLbu1_P_{lacA}_mCherry is shown in Fig. 2c.

For promoter characterization, mCherry expression under induced and non-induced conditions was monitored. Lactose as well as the non-metabolizable lactose analogues isopropyl-β-D-thiogalactopyranoside (IPTG) and thiomethyl-β-D-galactoside (TMG) were tested for induction of P_{lacA}. IPTG and TMG failed to induce LacR controlled gene expression (data not shown). This is in contrast to previous findings, where TMG was successfully used for the induction of ß-galactosidase expression in *L. plantarum* ATCC® 8014™ [19]. Different sugars were tested for induction of P_{lacA} (including lactose, xylose, fructose, glucose, maltose, arabinose and galactose), but P_{lacA} was only induced with lactose.

Lactobacillus plantarum 3NSH harboring plasmid pCDLbu1_P_{lacA}_mCherry were grown on selective media either containing 2 % glucose or 2 % galactose as carbon source and were induced with 0.5 or 2 % lactose after 2 h. Induction of mCherry expression with lactose was weak, but slight increase of RFUs was observed upon the addition of 0.5 or 2 % lactose on glucose (Fig. 4a), but was not observed on galactose (Fig. 4b). Contradicting the observation by Hasan and Durr [14], we did not detect full repression in the presence of glucose.

Catabolite inhibition through diminished entry of lactose into the cell could explain why calculated OD_{600} does not increase with additional carbon source (Fig. 4c, d). Negative control (without the promoter/repressor fragment; Fig. 4a, b, solid line) grew weaker on galactose without obvious reason.

Compared to the negative control and compared to growth on glucose, minor growth impairment of the plasmid containing cells on 2 % galactose (Fig. 4d) or 2 % lactose (Fig. 4f) as carbon source was observed. Figure 4e, f show mCherry expression and growth on selective MRS medium with 0.5 and 2 % lactose as the sole carbon source and inducer. In contrast to data presented in Fig. 4a, mCherry expression increases, showing the catabolite repressive effect of glucose and galactose on P_{lacA} or on cell entry of lactose. An increase from 0.5 to 2 % lactose increases expression (Fig. 4e) and growth (Fig. 4f). However, obtained calculated OD_{600} values on galactose and induction with lactose (Fig. 4c, f, filled circle) were comparable and did not increase, albeit the twofold amount of carbon source was available.

The chromosomally encoded *lac* locus (lp_3468, lp_3469 and lp_3470) as well as existence of a second *lac* locus (lp_3483, lp_3484), as indicated for *L. plantarum* WCSF1 genomic sequence [21], might interfere with usage of lactose as inducer, since *L. plantarum* 3NSH can utilize lactose as carbon source. The expression levels of mCherry under control of endogenous P_{lacA} were rather low on glucose or galactose, but when lactose was used as sole carbon source and inducer, expression levels improved significantly.

The synthetic promoter $P_{lacSynth}$ is inducible by IPTG

The *lac*A promoter and the *lac*I promoter/repressor are widely used for many different *E. coli* based expression systems and many mutant versions are available [48]. Therefore, we synthesized a DNA template consisting of the promoter P_{2083} of *L. buchneri* CD034 gene LBUCD034_2083 [16], containing two operator binding sites of the *E. coli* LacI repressor. We inserted a codon optimized version of the *E. coli* LacI repressor gene (Additional file 2: Figure S2) under control of the constitutive promoter P_{0234} of the *L. buchneri* CD034 gene LBUCD034_0234 [16]. Operator binding sites (O_1 and O_{id}) for Lac repressor binding were selected according to Oehler and colleagues [30] and integrated into P_{2083}. In *E. coli*, a third operator binding site (O_2) is encoded within the coding sequence of *lac*A [31]. This downstream *cis*-acting regulative sequence is involved in DNA bending and interaction with LacI multimers. But because integration of O_2 sequence into the mCherry coding sequence was not realizable, O_2 was not included in our constructs.

A synthetic regulative element for mCherry expression (P_{0234}_*lacI*_P_{2083}_*mCherry*) was constructed and promoter sequence and regulative elements are shown in Fig. 1c. LacI binding sites, operator binding sites (O_1 and O_{id}), RBS and P_{2083} are indicated. The consecutive construct is termed $P_{lacSynth}$ and cloned into pCDLbu1 (Table 2; Fig. 2a). The resulting expression vector pCDLbu1_$P_{lacSynth}$_mCherry is depicted in Fig. 2d.

According to *E. coli lac*-operon regulation, we tested mCherry expression subsequent to induction with IPTG. Increasing concentrations in the range of 0.1 to 5 mM (0.1, 0.5, 1.0, 2.0 and 5.0 mM) were tested and showed that already 0.1 and 0.5 mM induce $P_{lacSynth}$ sufficiently in *L. plantarum* 3NSH. Lower IPTG concentrations (like 0.1 and 0.5 mM) are in the range of common *E. coli* implementations. Moreover, 1 mM IPTG led to saturated induction of mCherry in *L. plantarum* 3NSH (Fig. 5a,

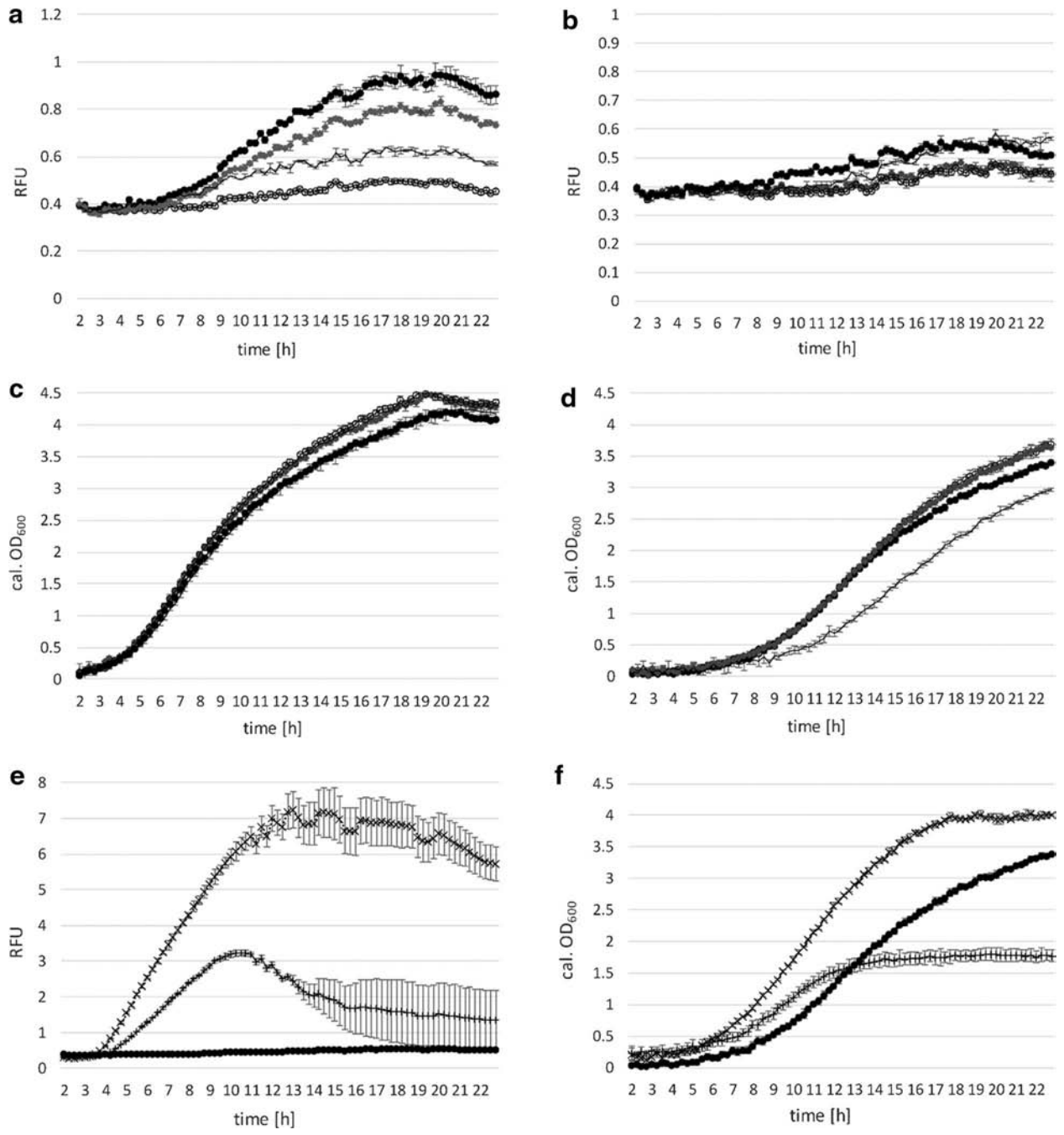

Fig. 4 Promoter characteristics of P_{lacA} and growth behavior. **a**, **c** MRS medium with glucose and 5 µg ml^{-1} CM; **b**, **d** MRS medium with galactose and 5 µg ml^{-1} CM; **e**, **f** MRS medium with 5 µg ml^{-1} CM. Mean values of four replicates are given and standard deviations are indicated. *Filled circle* induced, 2 % lactose. *Filled diamond* induced, 0.5 % lactose. *Circle* non-induced. **a**, **b** specific expression levels of mCherry under control of P_{lacA} after induction with 0.5 and 2 % lactose (or no induction) after 2 h in BioLector® micro-fermentation. Change of mCherry expression (RFUs, relative fluorescent units) over time (hours) of P_{lacA} mediated expression in comparison to the negative control is shown (*solid line*). **c**, **d** corresponding calculated OD_{600} values. **e**, **f** RFU and growth in selective MRS with lactose as main carbon source; x: induced, 2 % lactose; +: induced, 0.5 % lactose; *filled circle* induced, 2 % lactose and 2 % galactose as additional carbon source

b) and no further increase of expression was observable at augmented concentrations from 1 to 5 mM (data not shown). Additionally, TMG was tested for $P_{lacSynth}$ induction. We observed similar mCherry expression with induction of 17 mM TMG compared to 1 mM IPTG (data not shown). For *L. plantarum* NC2 it was shown

Table 2 Plasmids and strains used in this study

Plasmid	Reference	Size (bp)	Relevant characteristics	
pET-30a	Novagen	5400	T7 promoter, T7 terminator	
pE194	[18]	3728	Erythromycin resistance gene (ermE)	
pCD256	[43]	4790	Low copy plasmid in *L. plantarum*	
pCDLbu1	[15]	5776	High copy plasmid in *L. plantarum*	
pCDLbu1_P_{T7}_mCherry_T_{T7}_Ery	This study	6425	T7 RNA polymerase specific promoter	
pCDLbu1Δ*Ec*_P_{T7}_mCherry_T_{T7}_Ery	This study	3989	T7 RNA polymerase specific promoter, without sequences for replication and selection in *E. coli*	
pCDLbu1Δ*Ec*_P_{11}_mCherry	[47]	3809		
pCD256_$P_{lacSynth}$_mCherry	This study	6959	Low copy plasmid; promoter $P_{lacSynth}$; gene of interest mCherry	IPTG (1 mM)
pCD256_$P_{lacSynth}$_T7RNAP	This study	8882	Low copy plasmid; promoter $P_{lacSynth}$; gene of interest T7 RNA polymerase	IPTG (1 mM)
pCDLbu1_$P_{lacSynth}$_mCherry	This study	8164	High copy plasmid; promoter $P_{lacSynth}$; gene of interest mCherry	IPTG (1 mM), TMG (17 mM)
pCDLbu1_P_{lacA}_mCherry	This study	7917	High copy plasmid; promoter P_{lacA}; gene of interest mCherry	Lactose (0.5 -2 % *w/v*)
pCDLbu1_P_{xylA}_mCherry	This study	8077	High copy plasmid; promoter P_{xylA}; gene of interest mCherry	Xylose (0.2 -2 % *w/v*)
pCDLbu1_$P_{xylA(nativeRBS)}$_mCherry	This study	8077	High copy plasmid; promoter P_{xylA}; gene of interest mCherry; native RBS and spacer sequence from *B. megaterium* DSMZ 319 *xylA*	Xylose (2 % *w/v*)
Strains				
B. megaterium DSMZ 319	DSMZ			
E. coli Neb10β	NEB			
L. plantarum CD033	[43]			
L. plantarum 3NSH	[17]		Plasmid cured *L. plantarum* CD033	

that ß-galactosides are transported via ATP driven proton motive force [19]. Induction of recombinant gene expression in a fermentation setting (BioLector® measurement) with IPTG (and TMG) is shown here for the first time in *L. plantarum*.

Comparative growth and induction on selective media with glucose or galactose are shown in Fig. 5c and d. Induced cultures show growth impairment (compared to the non-induced cultures) on both carbon sources, though growth on glucose as carbon source is preferred, thus leading to higher expression values and biomass (Fig. 5a, c). Overall, regarding expression levels as well as repression under non-induced conditions in both tested media variations, $P_{lacSynth}$ performed better than P_{xylA} (Fig. 3) and P_{lacA} (Fig. 4). Negative control (without the promoter/repressor fragment) showed no mCherry expression (Fig. 5; solid line). Therefore, measured expression levels correlate to induction of $P_{lacSynth}$ through thiogalactosides, such as IPTG (and TMG), and basal expression might be caused by weak repression of P_{2083}.

Consequently, we suggest limited stoichiometric availability of the repressor LacI, resulting in incomplete repression of $P_{lacSynth}$ by the repressor. LacI binds to the operator sites by forming tetramers, which might not be possible if LacI availability is not sufficient [31, 49]. A stronger promoter for LacI expression (instead of P_{2083}) might increase repressor levels and improve transcription control. Additionally, the operator O_2 downstream of the start codon, which is originally present within the coding sequence of β-galactosidase [31], is absent within the mCherry sequence. Therefore, bending of DNA via binding of tetrameric Lac-repressor to two adjacent operators for sufficient repression is not possible. Albeit, it was reported for *E. coli* that the presence or absence of operator O_2 does not have an impact on *lac* operon expression anyhow [27].

Apparently, on selective medium with glucose as carbon source, the ratio of induced expression to basal expression under non-induced conditions of $P_{lacSynth}$ was highest compared to P_{xylA} or P_{lacA} (Figs. 3, 4). However, the $P_{lacSynth}$ mediated expression after induction is still moderate and thus appropriate for the regulation of T7 RNA polymerase based expression of mCherry. Therefore, this synthetic promoter/repressor fragment was used for establishment of the inducible T7 system in *L. plantarum* 3NSH.

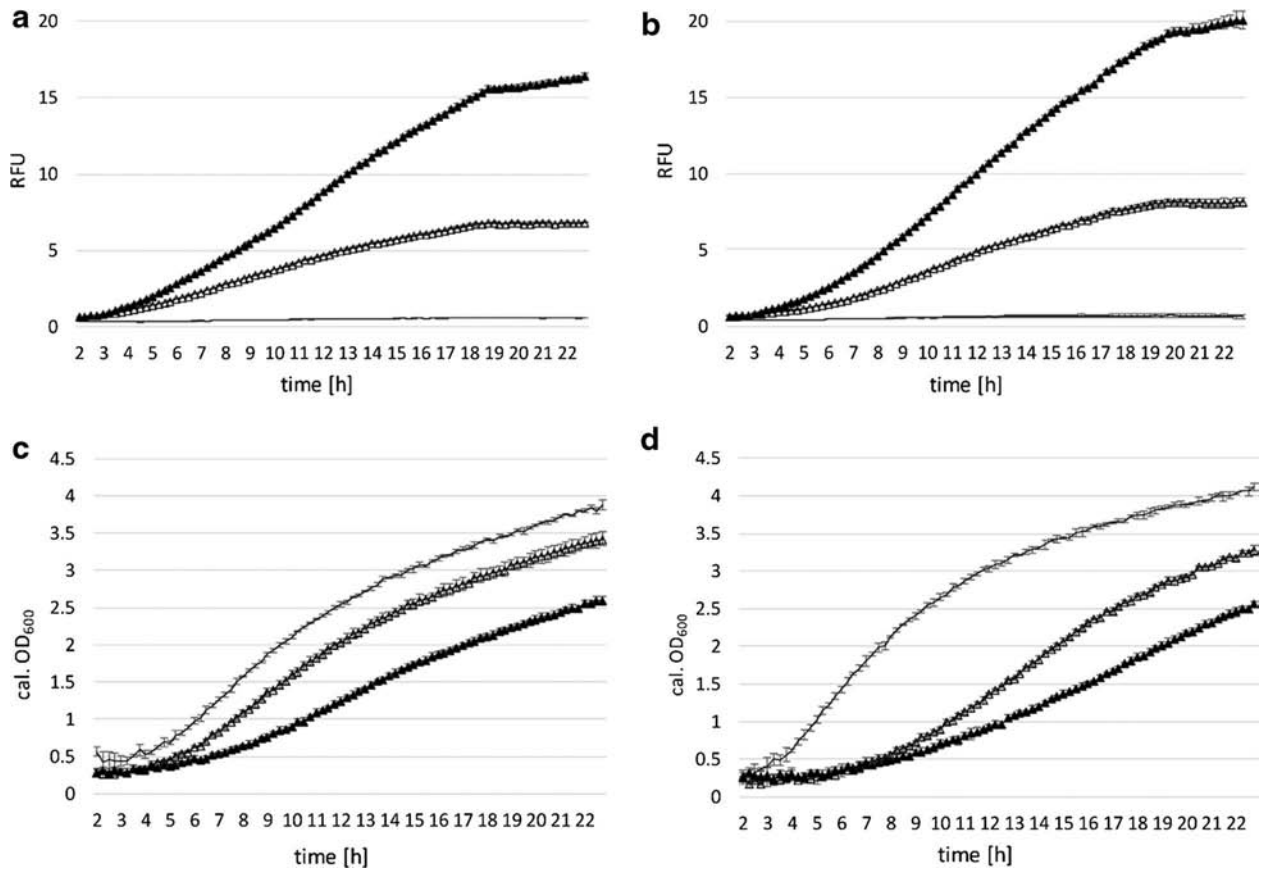

Fig. 5 Promoter characteristics of $P_{lacSynth}$ and growth behavior. **a**, **c** MRS medium with glucose and 5 µg ml^{-1} CM; **b**, **d** MRS medium with galactose and 5 µg ml^{-1} CM, mean values of four replicates are given and standard deviations are indicated. *Filled triangle* induced; *triangle* non-induced. **a**, **b** specific expression levels of mCherry under control of $P_{lacSynth}$ after induction with 1 mM IPTG (or no induction) after 2 h in BioLector® microfermentation. Change of mCherry expression (RFUs, relative fluorescent units) over time (hours) of $P_{lacSynth}$ mediated expression in comparison to the negative control is shown (*solid line*). **c**, **d** calculated OD_{600} values. *Solid line* negative control

T7 RNA polymerase driven mCherry expression in *L. plantarum* 3NSH

In order to establish an orthologous expression system in *L. plantarum*, we combined the synthetic repressor/promoter system $P_{lacSynth}$ (Fig. 1c). The adapted *E. coli* phage T7 RNA polymerase was applied to establish two compatible plasmids: one contained a codon optimized version of the T7 RNA polymerase (Additional file 3: Figure S3) under the control of $P_{lacSynth}$ (Fig. 2f) and the second plasmid contained mCherry under control of the T7 RNA polymerase promoter P_{T7} (Fig. 2g).

For inducing T7 RNA polymerase expression, we used 1 mM IPTG, equally to $P_{lacSynth}$ induction (Fig. 5). Results of the expression experiment are shown in Fig. 6a. Induction with 1 mM IPTG led to augmented expression of the reporter protein compared to non-induced conditions. Some basal expression of the reporter gene was detected under non-induced conditions similarly to results with $P_{lacSynth}$ (Fig. 5a). Therefore, the plasmid containing mCherry under control of the T7 RNA polymerase promoter (pCDLbu1Δ*Ec*_P_{T7}_mCherry_T_{T7}_Ery) was tested in absence of the second plasmid, which provides the T7 RNA polymerase (pCD256_$P_{lacSynth}$_T7RNAP). Thereby, we observed no mCherry expression neither with nor without IPTG (Fig. 6a, solid line).

Although, constitutive expression using the P_{11} promoter was significantly higher, inducible expression based on the T7 RNA polymerase system serves as a valuable tool for regulated gene expression at moderate levels. However, growth was not affected by $P_{lacSynth}$ regulating a dual plasmid expression system (Fig. 6b) compared to strains with plasmid pCDLbu1_$P_{lacSynth}$_mCherry (Fig. 5c). This effect could also be contributed to the different plasmid backbones (pCDLbu1 and pCD256, Fig. 2d, f).

For constructing the T7 polymerase encoding plasmid, the low copy plasmid pCD256 was used (Table 2). The second plasmid (mCherry under control of P_{T7})

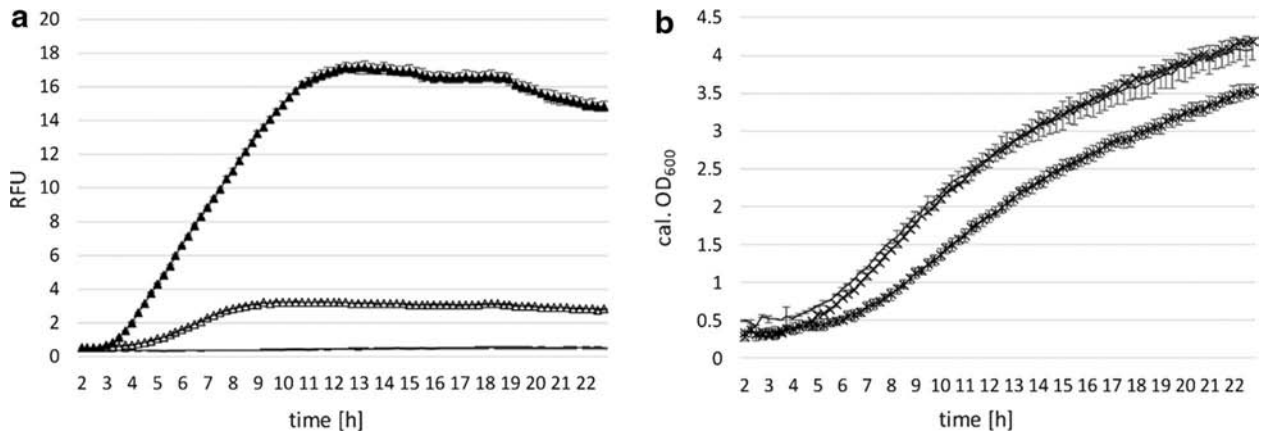

Fig. 6 Promoter characteristics and analysis of mCherry expression under control of P_{T7}, regulated by T7 RNA polymerase under control of $P_{lacSynth}$, and growth behavior. **a** Change of mCherry expression (RFUs, relative fluorescent units) over time (hours) regulated by T7 RNA polymerase dual plasmid system in BioLector® micro-fermentation is shown. T7 RNAP is under control of $P_{lacSynth}$ and induced with 1 mM IPTG (or non-induced) after 2 h. MRS medium with glucose either 5 µg ml^{-1} CM or 5 µg ml^{-1} Ery (or both for the dual plasmid system). –x-: induced; mean values of four replicates are given and standard deviations are indicated. –: non-induced; mean values of three replicates are given and standard deviations are indicated. **b** Calculated OD_{600} values. *Solid line* negative control (n = 3; pCDLbu1Δ*Ec*_P_{T7}_mCherry_T_{T7}_Ery)

was established from pCDLbu1 (Table 2) without *E. coli* specific sequences. Thereby we generated a smaller plasmid and less genetic load. Intentionally we introduced two different origins of replication within a cell, which is known to be preferred due to plasmid incompatibility [29]. Chromosomal integration of expression cassettes has been shown previously in *L. plantarum* [24, 36] and would be a feasible strategy for generating a stable T7 RNA polymerase expressing host strain. Such a *L. plantarum* strain would be the basis for a new T7 based expression system in a food grade host, providing specific regulation and easy exchange of any target gene that is under control of the T7 promoter P_{T7}.

Comparative analysis and semi-quantitative Western blot

The constitutive *L. plantarum* promoter P_{11} (expression vector pCDLbu1Δ*Ec*_P_{11}_mCherry) served as a benchmark in a comparative analysis [38, 47]. Plasmid pCDLbu1Δ*Ec*_P_{11}_mCherry is shown in Fig. 2e. Measurements of expression levels with plasmid pCDLbu1Δ*Ec*_P_{11}_mCherry were included for intrinsic comparison, because it was previously shown to yield strongest expression of mCherry amongst several tested variants in *L. plantarum* CD033 [47], the parental strain of *L. plantarum* 3NSH.

Expression levels of mCherry under control of P_{11} were compared to P_{xylA}, P_{lacA}, $P_{lacSynth}$, and the $P_{lacSynth}$ regulated T7 RNA polymerase, in Fig. 7a. Growth curves of producing strains and wild type are shown in Fig. 7b. MRS selective medium was used with galactose as carbon source and induction with xylose for pCDLbu1_P_{xylA}_mCherry, and with glucose plus induction with 2 % lactose for pCDLbu1_P_{lacA}_mCherry. Growth on glucose and induction with 1 mM IPTG was used for pCDLbu1_$P_{lacSynth}$_mCherry, and subsequently for the T7 dual plasmid system. P_{11} driven expression (pCDLbu1Δ*Ec*_P_{11}_mCherry) is more effective (Fig. 7a). Expression levels of P_{lacA} were quite low for direct comparison with promoter P_{11}, but results with P_{lacA} were also included in Fig. 7a and b.

A semi-quantitative Western blot of the inducible promoter systems at induced and non-induced conditions on glucose (Fig. 8a, c) or galactose (Fig. 8b) was performed. Sample point is indicated as vertical dotted line in Fig. 7 after 13 h of growth. About 5 µg biomass per slot were applied, the commercially obtained positive control (mCherry-His_6; 28.8 kDa) was applied at concentrations of 25 and 50 ng per slot. P_{11} samples were applied undiluted and 1:5 diluted due to stronger expression compared to the inducible systems (Fig. 7a). The Western blot shows better inducibility of the $P_{lacSynth}$ system on glucose medium (Fig. 8a) whereas P_{xylA} induction is more distinct on galactose medium (Fig. 8b) and the T7 system is inducible on glucose (Fig. 8c). We also observed basal transcription under non-induced conditions for all compared promoter/repressor systems. This is in accordance with the BioLector® measurements.

Suitable and controllable expression levels were achieved by $P_{lacSynth}$ induction with IPTG on glucose and galactose (Fig. 5a, b). Apparently, the ratio of induced expression to basal expression under non-induced conditions of $P_{lacSynth}$ on selective medium with glucose as carbon source was

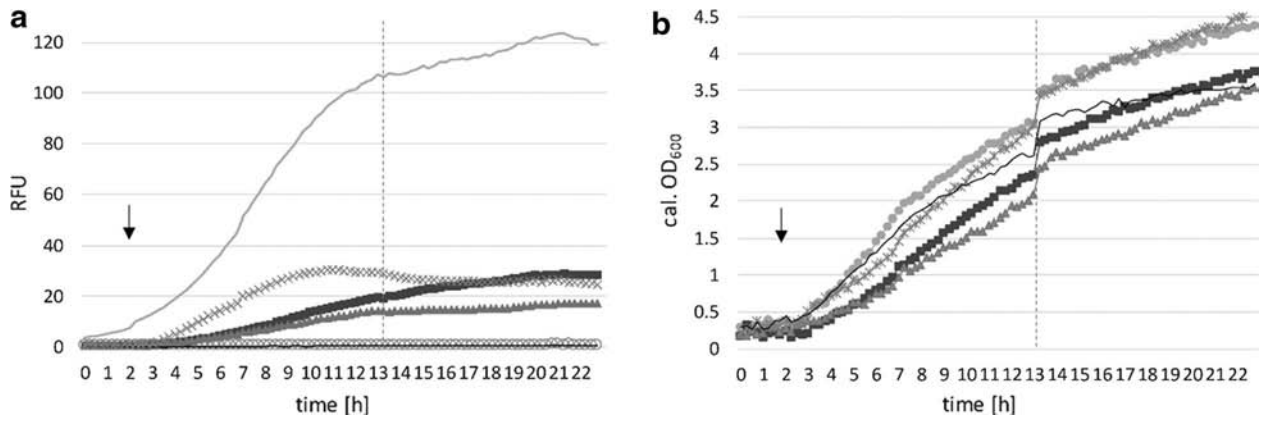

Fig. 7 Expression and growth comparison of inducible promoters to constitutive promoter. BioLector® micro-fermentation measurement for 23 h in selective MRS medium with glucose with low pH FlowerPlate at 30 °C (individual values). *Dotted vertical line* indicates sampling point (200 µl) for semi-quantitative Western blot, 13 h after start, followed by ongoing measurement, therefore OD_{600} curves are shifted after sampling. *Arrow* indicates induction time point (or absence of inducer). *Solid line* mCherry under control of constitutive P_{11} promoter (pCDLbu1Δ*Ec*_P_{11}_mCherry), –x-: T7 RNA polymerase based dual plasmid system induced with 1 mM IPTG, *square* P_{xylA} induced with 2 % xylose, *triangle* $P_{lacSynth}$ induced with 1 mM IPTG, *circle* P_{lacA} induced with 2 % lactose. **a** specific expression levels of mCherry under control of inducible promoters with respective inducers. **b** corresponding calculated OD_{600} values

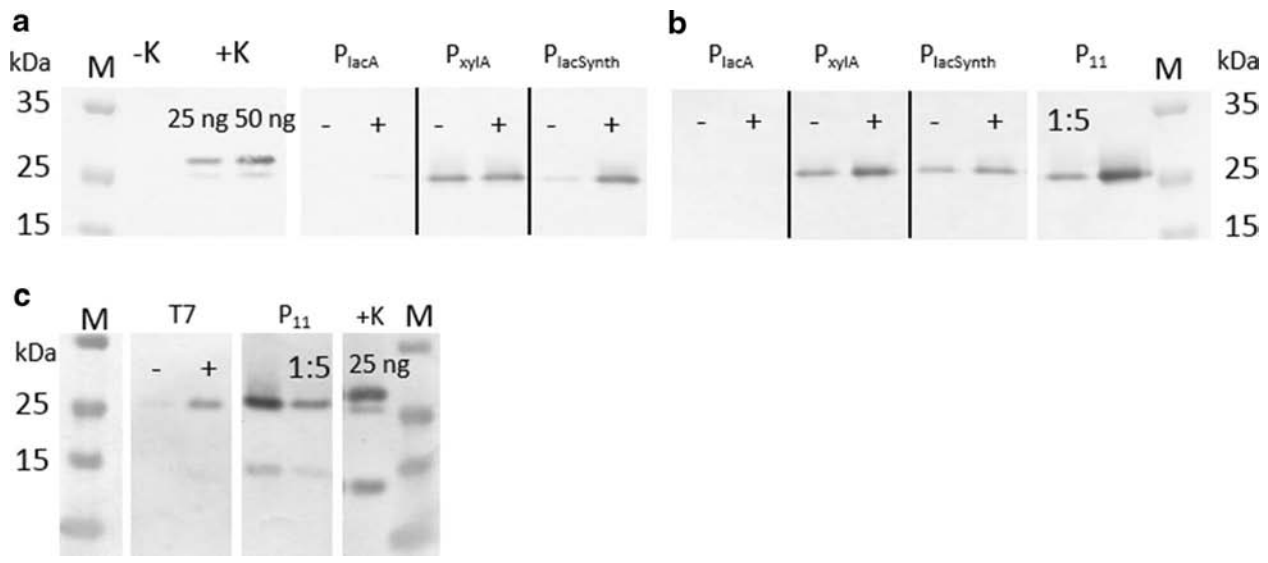

Fig. 8 Semi-quantitative Western blot analysis. Evaluation of intracellular mCherry (26.7 kDa) expression after 13 h of BioLecture® micro-fermentation measurement with low pH FlowerPlate at 30 °C. Five µg biomass per slot were applied. **a** MRS medium with glucose and 5 µg ml^{-1} CM; **b** MRS medium with galactose and 5 µg ml^{-1} CM; **c** T7 RNAP dual plasmid system, MRS medium with glucose and 5 µg ml^{-1} CM and 5 µg ml^{-1} Ery. − non-induced, + induced with the respective inducer. P_{11} sample (mCherry under control of constitutive P_{11} promoter) is additionally diluted 1:5 to adjust to expression levels of inducible promoters (in accordance BioLecture® results). *M* pre-stained protein ladder; +*K* positive control mCherry(His_6) 28.8 kDa; −*K L. plantarum* 3NSH wild type

best (compared to P_{lacA} or P_{xylA}). Moreover, the $P_{lacSynth}$ mediated expression after induction was still moderate and thus, more appropriate for the regulation of T7 RNA polymerase based expression of mCherry. By using a stronger promoter for *lacI*, expression, repression of $P_{lacSynth}$ might be improved. If necessary, this strategy could also be applied for *xylR* promoter and *lacR* promoter. Best regulation of induced and non-induced conditions while yielding similar expression levels was achieved by T7 RNA polymerase dual plasmid based system induced with 1 mM IPTG (Fig. 7a, 8c). The adapted T7 RNA polymerase was successfully established here for *L. plantarum*.

Conclusions

In this study, we tested and compared three different promoter-repressor systems for induced recombinant protein expression (red fluorescent protein mCherry) in plasmid free *L. plantarum* 3NSH. Reporter gene and regulatory elements were cloned into the high copy number plasmid pCDLbu1. The endogenous LacA promoter (P_{lacA}) derived from *L. plantarum* 3NSH showed only weak reporter gene expression upon induction with 2 % lactose, which was found to be the exclusive inducer so far. Glucose and galactose acted as repressors of P_{lacA}. With lactose as single carbon source better expression levels were obtained. The XylA promoter (P_{xylA}) derived from *B. megaterium* DSMZ 319 was tested in combination with the expression of the repressor XylR. Upon induction with 0.2–2 % xylose, we measured increased mCherry expression during exponential phase, and repression under non-induced conditions with galactose as the carbon source. A synthetic promoter ($P_{lacSynth}$), based on the *E. coli* derived *lac* operon resulted in moderate expression levels after induction with IPTG and TMG. $P_{lacSynth}$ was used efficiently for the establishment of a dual plasmid system for well-regulated T7 RNA polymerase expression, and transcription of mCherry under control of the T7 RNA polymerase promoter.

Some feasible suggestions for inducible recombinant protein expression in *L. plantarum* 3NSH are presented in this study. Expression levels of recombinant protein are however, much lower as compared to expression levels driven by the constitutive P_{11} promoter. Plasmid pCDLbu1Δ*Ec*_P_{11}_mCherry served as a benchmark and has been described previously [47]. Additionally, differences of expression in exponential phase, initiated by varying promoters, decreased during prolonged fermentation. In stationary phase (after 22 h) mCherry levels of tested inducible promoters are aligned. Yet, depending on the recombinant protein (e.g. amino acid composition and post-translational modifications) or experiment outlook (e.g. short time setting or production of cell toxic products) promoters, which are inducible by conventional sugars or well-established inducers are of particular interest. Although general knowledge of recombinant protein expression (e.g. therapeutics or metabolites) in lactobacilli steadily increases, efficiency and expression levels are not yet comparable to *E. coli* based systems. Recombinant gene expression usually exerts additional metabolic burden for the host. This often results in unstable genetic constructs, inhibition of cell growth and/or plasmid loss. Therefore, inducible expression systems where transcription of the target gene can be tightly controlled are preferable. The presented expression systems might behave different in other *Lactobacillus* strains, the adaption of new promoter/repressor systems, and in particular a T7 RNA polymerase based expression systems for *L. plantarum,* is anticipated to contribute to a flexible genetic tool box for cell engineering and recombinant protein expression in lactic acid bacteria.

Methods

Enzymes and gene synthesis

All restriction and modifying enzymes, as well as Q5 DNA polymerase, were purchased from New England Biolabs (NEB). Primers (Table 1) were obtained from Integrated DNA Technologies (IDT) and phosphorylated primers were synthesized by Sigma-Aldrich.

The ribosome binding site (RBS) was identical for all constructs. Identical Shine-Dalgarno sequence (SDS) and spacer region (bold) was selected for every construct according to SOPT#9 (5′-AAGGAGG**AAATTATA-C**ATG-3′), tested for efficient mCherry (start codon underlined) expression in *L. plantarum* CD033 [47].

Reporter gene mCherry and the synthetic LacR repressor/promoter fragment ($P_{lacSynth}$) were codon optimized for *L. plantarum* WCSF1 using http://www.jcat.de/ and synthesized by GeneArt® (life technologies). Promoter $P_{lacSynth}$, T7 RNA polymerase and transcriptional terminator from *L. buchneri* CD034 D-lactate hydrogenase gene (AFS00145.1) [16] were also codon optimized as described above and synthesized by GeneArt®. Nucleotide sequence of the codon optimized synthetic promoter/LacI repressor system is shown in Additional file 2 and T7 RNA polymerase is shown in Additional file 3.

Strains and cultivation conditions

Plasmids were constructed and propagated in *E. coli* Neb10β and clones were selected on LB agar plates with 100 μg ml^{-1} Ampicillin at 37 °C. Sequence positive plasmids were amplified for transformation into plasmid cured *L. plantarum* 3NSH [17]. Clones were selected on either MRS agar plates with either 5 μg ml^{-1} chloramphenicol (CM), 5 μg ml^{-1} erythromycin (Ery) or both combined at 30 °C.

In liquid medium, *E. coli* strains were cultivated under agitation at 37 °C in LB-medium. *L. plantarum* 3NSH was cultivated at 30 °C under oxygen limitation without agitation in MRS medium [7], supplemented with either 2 % (*w*/*v*) D-glucose or 2 % (*w*/*v*) D-galactose. *B. megaterium* DSMZ 319 was purchased from the "Deutsche Sammlung von Mikroorganismen und Zellkulturen" (Braunschweig, Germany) and was cultivated aerobically at 30 °C in nutrient medium. Antibiotics were added as required equally to solid media preparations.

Plasmid extraction was performed with plasmid purification kit for high-copy *E. coli* plasmids (NucleoSpin® Plasmid, Macherey–Nagel). After sequence verification, plasmids were used to transform *L. plantarum* 3NSH

[44]. Plasmid isolation from *L. plantarum* 3NSH was performed according to Sambrook and Russel [39] with addition of 10 mg ml^{-1} lysozyme (Merck, 105281) and RNase (R6513, Sigma) to the resuspension buffer and incubation for 30 min at 37 °C before cell lysis.

Construction of the P_{xylA}/xylR-plasmid

The *xylR* repressor/promoter fragment (P_{xylA}) was amplified from genomic DNA of *B. megaterium* DSMZ 319. Therefore, an overnight culture was used for DNA extraction, with pre-treatment described for Gram-positive bacteria (DNeasy Blood and Tissue Kit, Quiagen). Primers B_mega_XylOP_out_F and B_mega_XylOP_R(*Spe*I, *Sca*I, *Bam*HI) were used for amplification of the *xylR* repressor and *xylA* promoter genes with native RBS of *xylA*. Primers mCherry_w/o_RBS_*Xba*I and p256_miniori_for were used for amplification of reporter gene mCherry and Terminator T_{ldh} from *L. casei* BL23 (L-lactate dehydrogenase gene, LCABL-06930) from pCD256_$P_{lacSynth}$_mCherry (Fig. 2h). Constructs were ligated at *Spe*I and *Xba*I complementary overhangs and amplified via a PCR using B_mega_XylOP_F_*Mfe*I, *Kpn*I and Tldh_amp_*Pst*I_R, digested with *Kpn*I and *Pst*I and cloned into the pCDLbu1 plasmid (Fig. 2a) with an origin of replication for *E. coli* and *L. plantarum* [43].

For generating expression constructs with identical RBS and spacer sequence, we exchanged the native RBS of *xylA* (Additional file 1) with the RBS sequence SOPT#9 [47]. Nucleotide sequence of final P_{xylA} is shown in Fig. 1a. Therefore, we performed a continuous PCR around the ligated plasmid with phosphorylated primers B_mega_XylOP_newRBS_*Xba*I_Phos_R and mCherry_Phos_F. After ligation, plasmid pCDLbu1_P_{xylA}_mCherry (Fig. 2b) was transformed into *L. plantarum* 3NSH. For screening and sequencing purposes, primers B_mega_XylOP_seq_F and B_mega_XylOP_seq_R were used.

Construction of the P_{lacA}/lacR-plasmid

The plasmid pCDLbu1_P_{xylA}_mCherry (Fig. 2b) was *Sac*I and *Xba*I digested and fused with the LacR repressor/promoter fragment (P_{lacA}). This fragment was amplified from genomic DNA of *L. plantarum* 3NSH (DNeasy Blood and Tissue Kit, Quiagen) with the primers LacI_Lplant_F_*Sac*I and LacI_Lplant_R_*Xba*I and sequenced (sequence of P_{lacR} and P_{lacA} is shown in Fig. 1b). BLASTn analysis showed 99 % coverage (three mismatches) with the transcription regulator *lacR* gene of *L. plantarum* WCSF1. After ligation and transformation into *E. coli* Neb10β, sequence positive plasmid pCDLbu1_P_{lacA}_mCherry (Fig. 2c) was recovered and transformed into *L. plantarum* 3NSH. For screening and sequencing purposes, primers lacR_Gal_seq_R and lacR_Gal_seq_F were used.

Construction of the $P_{lacSynth}$/lacI-plasmid

Consecutive arrangement of synthetic $P_{lacSynth}$/LacI regulon (P_{0234}_*lacI*_P_{2083}) is shown in Fig. 1c. The promoter from *L. buchneri* CD034 gene LBUCD034_0234 [16] was selected for transcription of *lacI*, encoding the *E. coli* derived codon optimized LacI repressor (Additional file 2). The promoter from *L. buchneri* CD034 gene LBUCD034_2083 [16] was selected for regulation of the reporter gene mCherry. Operator binding sites [30] were artificially inserted into P_{2083} sequence. Operator sequence O_1 und O_{id} were adapted from *E. coli* [31]. Both constitutive *L. buchneri* CD034 promoters were identified within our group in previous promoter library experiments (data not shown).

The synthetic promoter was amplified from the synthetic GeneArt® construct with primers PlacSynth_F_*Sac*I_*Eco*RI and M13_R_*Nhe*I, digested with *EcoRI* and *Bam*HI and ligated into *Eco*RI and *Bam*HI digested pCD256 [43], receiving pCD256_$P_{lacSynth}$. This plasmid was amplified and proliferated in *E. coli*. Reporter gene mCherry was amplified from pCDLbu1Δ*Ec*_P_{11}_mCherry [47] with primers mCherry_RBS_*Xba*I and mCherry_R_*Bam*HI, digested and ligated with pCD256_$P_{lacSynth}$ (cut *Xba*I and *Bam*HI) plasmid. After transformation of *E. coli* and positive colony screening, pCD256_$P_{lacSynth}$_mCherry (Fig. 2h) was recovered and the insert was amplified via PCR with primers PlacSynth_F_*Sac*I_*Eco*RI and Tldh_amp_R_*Pst*I, followed by digestion with *Eco*RI and *Pst*I and ligation into digested pCDLbu1 (Fig. 2a) vector. The plasmid pCDLbu1_$P_{lacSynth}$_mCherry (Fig. 2d) was amplified in *E. coli* and subsequently introduced into *L. plantarum* 3NSH. For screening and sequencing purposes, primers mCherry_seq_R, mCherry_seq_F and Cat_seq 2_back were used.

Construction of the T7 RNA polymerase based dual plasmid expression system

DNA was amplified with the primers T7_RNAP_Lp_RBS and T7_RNAP_Lp_Term_R_*Sal*I from a synthetic template. The fragment was *Xba*I and *Sal*I digested and ligated into the *Xba*I and *Sal*I digested pCD256_$P_{lacSynth}$_mCherry plasmid (Fig. 2h), thus receiving the plasmid pCD256_$P_{lacSynth}$_T7RNAP (Fig. 2f).

The second plasmid (pCDLbu1Δ*Ec*_P_{T7}_mCherry_T_{T7}_Ery, Fig. 2g) was cloned stepwise. The reporter gene mCherry was amplified with primer M13_2_F and mCherry_R_*Bam*HI from plasmid pCDLbu1_$P_{lacSynth}$_mCherry (Fig. 2d), digested with *Xba*I and *Bam*HI and ligated into digested pET-30a plasmid (Table 2). Primers T7-Promoter_*Sac*I and T7-Terminator_*Sal*I were used to amplify the P_{T7}_mCherry_T_{T7} fragment from the established pET30a-mCherry plasmid. The erythromycin

resistance gene (*ermE*) was amplified with primers ery_*Kas*I_back and oripE194_seq_back from pE194 [18]. The *ermE* fragment was digested with *Cla*I to fuse it with the *Cla*I digested P_{T7}_mCherry_T_{T7} fragment, followed by an enrichment PCR with primers ery_*Kas*I_back and T7-Terminator_*Sal*I. The resulting fragment was digested with *Kas*I and *Bsp*EI and ligated into the *Kas*I and *Xma*I digested plasmid pCDLbu1 [15], resulting in the plasmid pCDLbu1_P_{T7}_mCherry_T_{T7}_Ery. After amplification of the plasmid in *E. coli* JM109, *E. coli* specific sequences (pMB1 origin of replication and ampicillin resistance gene) were removed by PCR with primers Ery_F_*Nhe*I and M13_R_*Nhe*I. The PCR product of plasmid pCDLbu1Δ*Ec*_P_{T7}_mCherry_T_{T7}_Ery (Fig. 2g) was digested with *Nhe*I, circularized by ligation and directly used to transform *L. plantarum* 3NSH [44].

Subsequent to sequence verification of a colony harboring plasmid pCDLbu1Δ*Ec*_P_{T7}_mCherry_T_{T7}_Ery was used for establishing competent cells and transformed with plasmid pCD256_$P_{lacSynth}$_T7RNAP, resulting in a strain carrying two different expression vectors. After transformation, cells were selected on MRS plates with 5 µg ml^{-1} CM and 5 µg ml^{-1} Ery. Colonies were screened for both expression plasmids verified by sequencing.

Construction of negative controls

For the inducible promoter/repressor constructs, a negative control plasmid was established by removing the whole inserted promoter/repressor fragment (plasmid with mCherry coding sequence and terminator; termed empty). The plasmid backbone, which is identical for every construct, was amplified with primers P_{empty}_*Sac*I_R and mCherry_RBS_*Sac*I_F from pCDLbu1_$P_{lacSynth}$_mCherry. The thereby established plasmid pCDLbu1_X_mCherry allows testing for mCherry expression, driven by possible read through from upstream regulatory sequences or possible unknown upstream promoter sequences.

As negative control for the T7 RNA polymerase dual-plasmid system, we used a clone harboring only plasmid pCDLbu1_P_{T7}_mCherry_T_{T7}_Ery. Thereby we tested if any other factors except T7 RNA polymerase contributes to mCherry expression.

Induction conditions

Over-night cultures were adjusted to OD_{600} 0.2 in the respective liquid medium. After 2 h of growth at 30 °C in the BioLector® micro-fermentation system, cultures were induced with the respective inducer 1:10 into each well, thus requiring that preparations of each inducer is tenfold concentrated in MRS-medium. Non-induced cells were prepared and tested simultaneously, but without the inducer (MRS medium only).

Tested sugars were used in the D(+)-configuration and weighted as solids (weight) per volume medium (*w*/*v*). The promoter P_{xylA} was induced with xylose. Either 0.2, 1 or 2 % xylose were used for induction. Therefore, 100 or 200 g l^{-1} D-xylose was added to the medium (MRS 5 µg ml^{-1} CM without glucose), heated in a water bath and sterile filtrated (0.2 µm) and diluted accordingly. Other preliminary tested sugars such as fructose, arabinose, maltose, as well as galactose were prepared likewise.

The synthetic promoter $P_{lacSynth}$ was induced with IPTG (VWR) and TMG (M8146, Sigma). Standard final concentration for IPTG was 1 mM. Therefore, 10 mM IPTG was dissolved in selective MRS medium and 1:10 diluted into respective wells. For testing minimum and maximum induction concentrations, we used dilutions ranging from 0.1 to 5 mM IPTG per well (0.1, 0.5, 1, 2 and 5 mM). 17 mM TMG was also tested for induction of $P_{lacSynth}$ in selective MRS medium, as well as 2 % lactose. For P_{lacA} standard conditions were selective MRS medium with 2 % glucose, 2 % maltose or 2 % galactose or without additional carbon source and induction with 0.5 or 2 % lactose after 2 h.

BioLector® and Tecan reader measurements of intracellular mCherry expression

Pre-measurements were performed in an Infinite® M1000 PRO Tecan microplate reader as described elsewhere [47]. The BioLector® micro-fermentation system (m2p-labs Germany) was also used for online measurement. Overnight cultures (in selective MRS medium with glucose or galactose) were diluted to an OD_{600} of 0.2 in the respective liquid medium. 720 µl of each sample were pipetted per well of MTP-48-BOH FlowerPlate® (low pH, Lot No. 1408) or MTP-48-B FlowerPlate® (without optodes, Lot No. 1402) in quadruplicates and sealed with sterile tape adhesive sealing (Nunc, 732-2610). Samples were induced after 2 h of growth. Under sterile conditions 80 µl of the particular inducer (tenfold concentrated in MRS medium) was pipetted into the respective well. 80 µl MRS medium were added to non-induced samples and controls, and plates were covered again with sealing tape. Results were analyzed after 23 h using the BioLection 2.3.13 software using a previously described calibration curve for *L. plantarum* [47]. Calibration parameters were set for 30 °C according to the manufacturer's recommendations.

SDS-PAGE and Western blot analysis

For Western blot analysis of intracellular mCherry (to compare the expression levels of under induced and non-induced conditions in selective MRS medium with glucose as carbon source) cells were collected at late

exponential phase after 13 h of growth (dotted vertical line in Fig. 7). Recombinant purified mCherry with His_6-tag (28.8 kDa) was purchased from BioVision (4993-100) and used as a positive control in defined concentrations per slot (25 and/or 50 ng). Per slot we applied samples corresponding to 5 µg biomass each (calculated as described below). The reference strain (pCDLbu1Δ*Ec*_P_{11}_mCherry) was applied undiluted and 1:5 diluted for adaption to mCherry yields obtained by induction of the inducible promoters.

The pellet of 200 µl culture was washed with PBS, centrifuged and pellet was re-suspended in 200 µl PBS. OD_{600} was measured of each sample. For analyzing equal amount of biomass $0.4/OD_{600}$ for each sample was calculated and used for intracellular analysis. A spatula tip of zirconium beads (BMBZ 100-250-17) was added to each sample, followed by alternating 30 s vortex and 30 s on ice; repeated for ten times. To remove cell debris and beads, samples were centrifuged at 4 °C full speed and supernatant was transferred into a fresh tube. A volume of 15 µl of each sample were mixed with 2 × LDS loading buffer and incubated at 99 °C for 10 min. Afterwards, 15 µl per sample and 5 µl protein ladder (Fermentas, SM0671) were loaded onto a NuPAGE® 12 % BisTris gels and electrophoresis was run with MOPS buffer. The gel was blotted onto a PVDF membrane. Anti mCherry antibody (Biovision, 5993-100; 1:10.000) and AP-linked anti-rabbit secondary antibody (Sigma A9919; 1:20.000) were used for detection of mCherry. BCIP/NBT Color Development Substrate (Promega, S3771) was used for staining the blot.

Additional files

Additional file 1: Figure S1. Comparison of RBS and spacer sequence of $P_{xylA\ (native\ RBS)}$ and P_{xylA}.

Additional file 2: Figure S2. Sequence of the codon optimized version of the *E. coli lacI* repressor gene and P*lacSynth*.

Additional file 3: Figure S3. Sequence of the codon optimized version of the T7 RNA polymerase gene.

Authors' contributions

The work presented here was carried out in collaboration between all authors. S Heinl, RG and S Heiss defined the research theme and designed the experiments. S Heiss, AH, CT, MS and EE carried out the laboratory experiments. S Heiss analyzed the data, interpreted the results and prepared this manuscript with input, feedback and advice from S Heinl and RG. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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5 Summary, Conclusions and Perspectives

L. plantarum CD033 (accession number BT6326; deposited in the strain collection of the Institute of Molecular Biotechnology, Graz, Austria) was isolated from a stable grass silage (Spath et al., 2012b) and inherently harbors a 7.8 kb plasmid pCD033.

In the first part of this thesis, the single plasmid of *L. plantarum* CD033 (pCD033) was sequenced and analyzed. 14 ORFs were identified whereof eight were predicted as CDS (coding sequences) due to the presence of a ribosomal binding site and/or similarity of the translated nucleotide sequence to already known proteins. We compared CDS1 gene product (RepB/OrfX-like protein) to related sequences of other plasmids, thus showing a conserved DGQHR domain. Additionally, we compared pCD033 to related plasmids with Artemis Comparison Tool and we defined the minimal stable replicon of pCD033, which is useful for the establishment of novel expression vectors for LAB. General information about pCD033 replication and stability were obtained, as well as determination of plasmid copy number (approximately 3 copies per cell). Absence of a replication initiation protein, a double strand origin as well as a single strand origin on plasmid pCD033 suggests replication via theta mechanism. We proposed a new type of plasmid replication, potentially initiated and regulated by non-coding RNA. Up to now, only one other *L. plantarum* plasmid is described having a similar mechanism (Sørvig et al., 2005), where no plasmid encoded protein seems to be involved in replication.

According to sequencing and annotation data no potentially essential genes are encoded on plasmid pCD033, but a PemI/PemK-like (antitoxin/toxin) plasmid addiction system is present (ORF5 and ORF6). A plasmid free strain is advantageous e.g. because of reduced genetic load. Therefore, we intend to cure *L. plantarum* CD033 of its native plasmid. Different curing strategies were tested and combined, such as using the pCD033 origin of replication. It is known that if similar origins of replication occur within one cell, loss of plasmid is enhanced due to plasmid incompatibility (Novick, 1987). Novobiocin as curing agent is well established (Bringel et al., 1989; Karthikeyan and Santosh, 2010; Ruiz-Barba et al., 1991). The PemI/PemK-like plasmid maintenance system interferes with curing, therefore, we applied a novel curing strategy. Thereby we introduced the anti-toxin gene *pemI* in *trans* and used oripCD033 in combination with novobiocin. By this successful strategy, the plasmid free

strain *L. plantarum* 3NSH was generated and used for further experiments. Previously, a similar but simpler approach has been used for *L. salivarius* (Fang et al., 2008). This manuscript is therefore extending methodologies and basic knowledge for *Lactobacillus*.

In another study, we tested constitutive promoters for recombinant protein production and established respective expression plasmids. Amongst others, we tested the endogenous P_{tuf33} and P_{tuf34} promoters. Promoter P_{11} (Rud et al., 2006) was used as benchmark and reached the highest expression levels, followed by P_{tuf34} and P_{tuf33}. Additionally, the number of nucleotides of the spacer sequence between the ribosomal binding site and translation start codon was varied and the optimal sequence for mCherry expression in *L. plantarum* CD033 was determined.

Moreover, the identification and characterization of inducible promoters for *L. plantarum* was in the focus of our interest. P_{11} promoter was also used as a reference in the following study. Only few regulable systems are described for lactobacilli (Axelsson and Holck, 1995; Böhmer et al., 2013; de Ruyter et al., 1996; Maidin et al., 2014), thus, there is strong demand for controllable heterologous gene expression. We therefore tested and characterized specific promoters in regard to mCherry expression. The novel plasmid free strain *L. plantarum* 3NSH was used and expression level were monitored with the BioLector® micro-fermentation system.

A xylose inducible promoter and its repressor from *Bacillus megaterium* DSMZ 319 were studied. Induction was only achieved with xylose whereas glucose was found to be a catabolite inhibitor. Already low xylose amounts were sufficient for induction on MRS medium with galactose as main carbon source. Furthermore, we characterized the endogenous *lacA* promoter and its repressor. mCherry expression was best when lactose was used as sole carbon source and no glucose or galactose was present in MRS-medium. Another object of our studies was the IPTG and TMG inducible *E. coli lacI* promoter and its repressor. The repressor was modified for Gram-positive bacteria regarding codon usage. In another orthologous approach we used this promoter/repressor system for regulation of the T7 RNA polymerase in a dual plasmid system, which was established successfully for *L. plantarum* 3NSH. Apparently, we mostly observed low basal expression also in absence of inducer for all tested constructs. Irrespective of relatively low mCherry yields (compared to the constitutive P_{11} promoter), we hereby extend the toolbox for recombinant protein expression in *L. plantarum*.

Altogether, we generated relevant data for controlled recombinant gene expression. A set of useful expression vectors could be established and the plasmid free strain *L. plantarum* 3NSH enhances applicability of these systems. This thesis presents a straightforward and innovative approach for improvement of *L. plantarum* as cell factory. A generally regarded as safe organsim is per se of interest for biotechnological applications and food-grade inducible promoters further enhance usability and perspective of *L. plantarum*. Furthermore, novel data contributes to general knowledge of Lactobacilli and presents a flexible toolbox for recombinant protein expression in lactic acid bacteria.

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6 Abbreviations

cfu	colony forming units
kbe	Kolonie bildende Einheiten
IPTG	Isopropyl β-D-1-thiogalactopyranoside
TMG	Methyl-β-D-thiogalactoside
MRS-medium	de Man, Rogosa and Sharpe Medium
ORF	open reading frame
CDS	coding sequence
LAB	lactic acid bacteria
GRAS	generally regarded as safe

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Project Work	2010-2011	**Cell engineering of protein producing yeast *Pichia pastoris***
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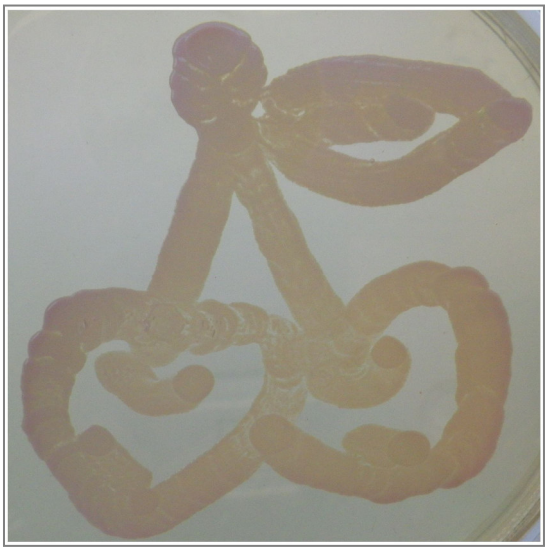

Merci Chérie