Investigation of storage polysaccharide metabolism in lactic acid bacteria

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Abstract

Glycogen biosynthesis and its regulation have been investigated extensively in bacteria at the level of ADP-Glc synthesis. The enzyme ADP-glucose pyrophosphorylase (ATP: α-dglucose-1-phosphate adenylyltransferase; EC 2.7.7.27; ADP-Glc PPase) catalyzes ADP-Glc formation in a reversible reaction which is the rate determining step of glycogen biosynthesis. This enzyme is encoded by *glgC* in all bacteria. However, the role of an additional, similar gene *glgD* in Gram-positive bacteria including lactic acid bacteria (LAB) is poorly understood. Only a regulatory role of *glgD* has been reported in *Bacillus stearothermophilus* without any activity being shown for the protein product GlgD. Therefore, it was decided to investigate the function of *glgC*-homologous to *glgD* gene in LAB. The focused new role of *glgD* might reveal the mechanism of regulation of glycogen in this group of bacteria. And possibility link intracellular polysaccharide (IPS) formation popular probiotic characterisation of certain species of LAB.

Our studies encompassed the LAB which are a group of facultative anaerobic, non-pathogenic, non-colonizing, non sporulating Gram-positive bacteria. The genes for glycogen synthesis have been found in the operon *glgBCDAP* or *glgCDAP-B* as in *Lactobacillus plantarum* WCFS1 and *Lactococcus lactis* subsp. *cremoris* MG1363, respectively. We have focused on the detailed functional analysis of both the genes *glgC* and *glgD* in *L. lactis* subsp *cremoris* MG1363 and SK11 and *Lb. plantarum* WCFS1. In a fusion expression study of both the genes *glgC* and *glgD* from *Lb. plantarum* WCFS1, we have shown experimentally that the proteins GlgC and GlgD have interact with each other. Both the proteins appear to be subunits forming the fully active enzyme with an α/β heteroligomer type structure. To our knowledge, this is the first study to prove that the GlgC & GlgD proteins have an interaction in lactic acid bacteria. Moreover, we have also observed a low enzymatic activity of the the GlgD protein in presence of ATP and a high affinity for UTP. The first observation suggests that GlgD has ADP-Glc-PPase activity in addition to GlgC and the second is indicative of an alternative reaction to produce UDP-Glucose possibly providing to an alternate pathway for glycogen biosynthesis.

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Abbreviations

AA	amino acid
Ap	ampicillin
APS	ammonium persulfate
ATP/ADP	adenosine 5'-triphosphate /Adenosine 5'-diphosphate
B. subtilis	*Bacillus subtilis*
BHI	brain-heart-infusion (medium)
bp	base pair
BSA	bovine serum albumin
C. glutamicum	*Corynebacterium glutamicum*
Cm	chloramphenicol
Da	Dalton
dd H_2O	double distillated water
DNA	Deoxyribonucleic acid
DNSA	3,5-Dinitrosalicylic acid assay
dNTP	deoxynucleosidetriphosphate
DSMZ	German Collection of Microorganisms and Cell Cultures
DTT	Dithiothreitol
E. coli	*Echerichia coli*
EC	Enzyme Commission
EDTA	ethylene di-amine tetra-acetic acid
e.g.	For example
EtOH	ethanol
EtBr	Ethidiumbromid
FPLC	Fast Protein Liquid Chromatography
h	hour
i.e.	That is
IPTG	Isopropyl ß-D-1-thiogalactopyranosid
Kb	kilobase pair
kDa	kilo Dalton
LAB	lactic acid bacteria
LB	Luria-Bertani Broth
Lb. plantarum	*Lactobacillus plantarum* WCFS1
L. lactis	*Lactosossuc lactis* subsp. *cremoris*
L	liter
NCIMB	National Collection of Industrial and Marine Bacteria, UK),

Abbreviations

NICE	nisin-controlled gene expression system
MBP	maltose binding protein
min	minutes
MS	Mass Spectrometry
OD	Optical Density
ON	overnight
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
P_i	inorganic phosphate
rpm	rounds per minute
RNA	ribonucleic acid
rRNA	ribosomal RNA
RT	room temperature
s/sec	seconds
SDS	sodium dodecylsulfate
TAE	Tris-Acetate-EDTA buffer
TE	Tris/EDTA buffer
TLC	thin layer chromatography
Tris	tris-hydroxymethyl-aminomethane
TEMED	N,N,N',N'-Tetramethylenethylendiamin
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
X-Gal	5-Bromo-4-chloro-3-indolyl-ß-D- galactopyranoside

Introduction

1.1 Lactic acid bacteria

Lactic acid bacteria (LAB) are metabolically and physiologically related group of Gram-positive bacteria which consist of both cocci and rods-shaped bacilli forms. Generally members of the LAB family are non-motile, non-spore-forming and are catalase negative. All of them grow anaerobically, though they are also able to grow in the presence of O_2 as “aerotolerant”. The LAB typically lack a respiratory chain and they are able to ferment a variety of sugars to produce lactic acid as the sole or major product along with energy required for cellular maintenance and growth. Therefore, based on their mode of sugar fermentation, they fall into two large metabolic categories. The first of thes,e produce lactic acid as the sole end product of hexose fermentation via glycolysis following the familiar Embden-Meyerhof-Parnas pathway (EMP) and are indicated as the homolactic fermentatives (homofermentatives LAB). The second category is designated as the heterofermantatives LAB which produce significant amount of other end products such as acetate, ethanol and CO_2 besides lactate. The apparent difference on the enzyme level between these two categories is the presence or absence of the key cleavage enzymes of the EMP pathway (fructose1, 6 diphosphate adolase) and Pentose phosphate (PK) pathway (phosphoketolase) Fig.1

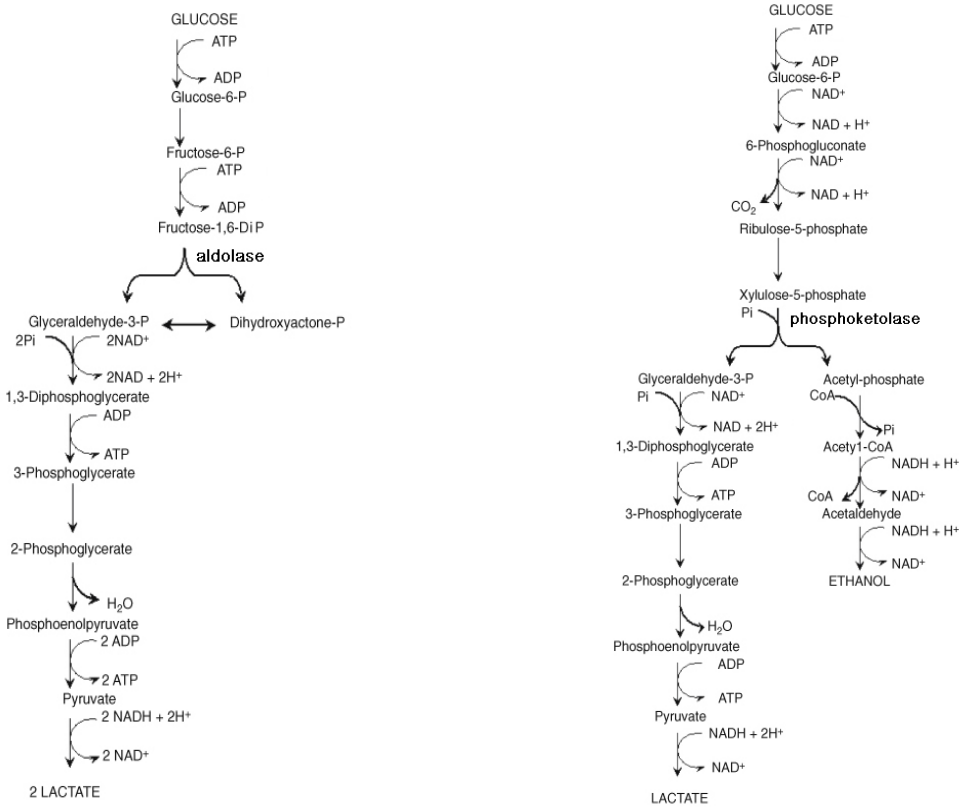


Fig.1.The pathway of homolactic and heterolactic acid fermentation in lactic Icid bacteria (courtesy: Todar´s online Textbook of bacteriology, Kenneth Todar)

Introduction

The traditional classification of LAB into different genera is based on the temperature of growth, mode of glucose fermentation, morphology, pH requirement, amongst other factors. With the advent of nucleotide sequencing, classification based upon 16S and 23S ribosomal RNA (rRNA) sequence data and G+C content (the percentage moles of guanine plus cytosine content in the genomic DNA), the Gram-positive bacteria form two lines of descent (Fig. 2; Stackebrandt & Teuber, 1988)). The LAB have a DNA base composition of less than 50 % G+C, and so are phylogenetically included in the *Clostridium* branch of Gram-positives. Nowadays, LAB comprise around 20 genera, of which *Aerococcus*, *Enterococcus*, *Carnobacterium*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissela* are considered as the principal LAB associated with foods (Axelsson, 2004).

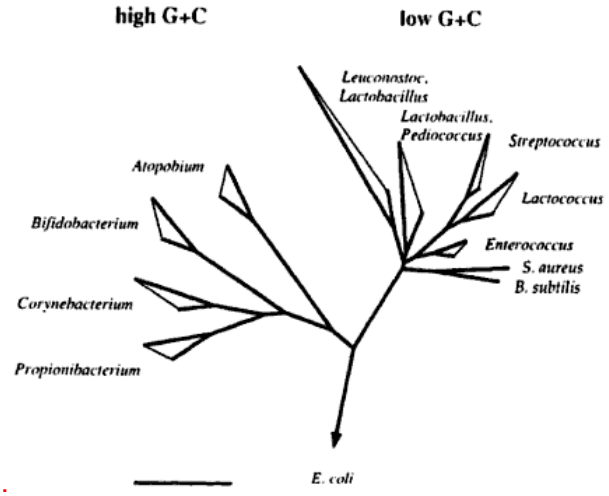


Fig. 2 Phylogenetic trees of Gram-positive bacteria

However, organisms such as those belonging to the genera *Listeria* and *Staphylococcus*, fermenting sugars with the production of lactic acid, are closely related to LAB by 16S rRNA sequences, except that they are catalase positive.

There are also other Gram-positive bacteria like *Corynobacterium*, *Microbacterium* and *Propionibacterium* that are important for food and feed production and are members of the Actinomycetes branch (Fig. 3; Schleifer & Ludwig, 1995).

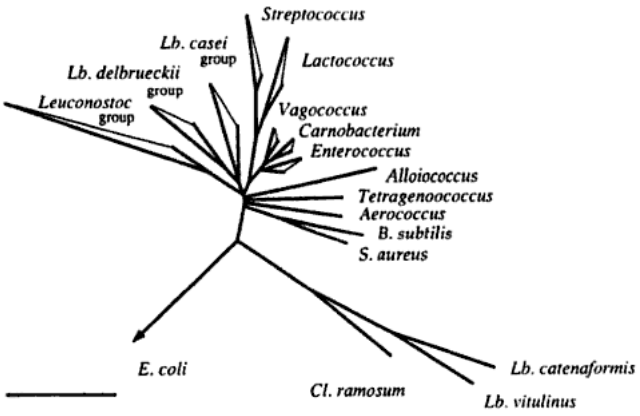


Fig. 3 Phyologenetic trees of the lactic acid bacteria and related bacteria

The LAB are capable of growth over a wide pH range and are characterized as “acidotolerant”. This is because the organic acid lactate produced by the LAB causes significant change of the environment by decreasing the pH thereby making the environment selective against less acid tolerance for other bacteria. The LAB are generally used in industrial food and fodder production.

Food–grade LAB are mostly from the *Lactococcus* and *Lactobacillus* family and are considered as non-pathogenic. LAB strains are usually used in the food fermentation as a starter culture especially for fermented dairy products, meat and vegetables. They have beneficial health effect on the host and also contribute to improve the food safety by providing preservation of the products. Many LAB strains were recognized and grouped under probiotic bacteria which are live microoganisms which when administrered in adequate amounts confer a health benefit on the host as defined by FAO/WHO and are Generally Regarded As Safe (GRAS status). Thus, many LAB strains are utilized as delivery vehicle of antigens for vaccines (Wells and mercenier, 2008) and to prevent growth of pathogens in food products (cavadini *et al*., 1998; Turner *et al*., 2007) as well as to develop many expression system such as inducible promoter, modified secretion signal sequences (Dieye *et al*., 2001; Ravan *et al*., 2003) for producing and secretion of heterologous proteins which are usually difficult to express in other prokaryotes. The most inducible expression system for LAB is the nisin controlled expression system NICE (de Ryter *et al*., 1996) where *nisA* promoter is induced by nisin antimicrobial peptide through two-component regulation system and the comparable inducible expression system using *pstF* promoter (Siren *et al*., 2008)

Introduction

1.1.1 *Lactococcus*

The laboratory strain *Lactococcus lactis* subsp. *lactis* IL1403 was the first lactic acid bacterial genome to be completely sequenced (Bolotin *et al*., 2001).
Lactococci are mesophilic lactic acid bacteria that was first isolated from green plants (fruits, vegetables, and cereal grains). This group of bacteria, previously classified with the lactic streptococci family (*Streptococcus lactis* subsp. *lactis* or *cremoris*) was placed by Schleifer in 1985 in the new group. *Lactococcus* family acquired much interest because many of their functional importance for successful fermentations are linked to plasmid DNA, which are commonly exchanged between strains via conjugation (McKay *et al*., 1985; Dunny and McKay, 1999). These bacteria are selected for use in the fermentations based on their ability to produce unique compounds (often from amino acid catabolism), resistance to bacteriophage and metabolic stability. Advances that define the fundamental knowledge of the genetics, molecular biology, physiology, and biochemistry of Lactococci will provide new insights and applications for these bacteria.

The important lactococci, *Lactococcus lactis* is a fermentative lactic acid bacterium that is used extensively in food fermentations and has great biotechnological and economic importance. *L. lactis* is one of the main bacteria used in starter cultures by the dairy industry for the production of cheese, fermented milks and their derivative.. Especifically *L. lactis* subsp. *cremoris* is known by its continual use in food fermentations (Beimfohr *et. al*., 1997; Garvie *et al*., 1981). *L. lactis* subsp. *cremoris* strains are preferred because of their superior contribution to product flavor via unique metabolic mechanisms over *L. lactis* subsp. *lactis* strains (Sandine. 1988; Salama *et al.,* 1991). The DNA sequence divergence between the subspecies is estimated to be between 20 and 30% (Godon *et al*., 1992). Of the many lactococcal strains, *L. lactis* subsp. *cremoris* SK11 strains are known to harbor many plasmids carrying industrially important traits which are recognized for the formation of beneficial flavor and aroma compounds (Lawrence *et al*., 1976). *L. lactis* subsp. *cremoris* SK11 is a phage-resistant strain widely used by the dairy industry.. The genome of the two *L. lactis* strains namely *L. lactis* subsp. *cremoris* SK11 and MG1363 have been completely sequenced. The sub-species of the genome of *L. lactis* subsp. *cremoris* MG1363 is 160-Kbps and 90-kbps larger than *L. lactis* subsp. *lactis* IL1403 and *L. lactis* subsp. *cremoris* SK11, respectively. Thus, 465 and 346 genes encoding those region are absent in the latter strains (Wegmann *et al*., 2007). About 47% of those genes were annotated to be used for transport and carbohydrate metabolism.

1.1.2 Lactobacillus

The genera Lactobacillus are the biggest in the family of LAB. Lactobacillus species are found in a variety of habitats rich in carbohydrate such as mucosal membranes of human as well as in animals (oral cavity, intestine and vagina) as well as on plant materials. They are commonly used to ferment food product and for preservation of food and fodder such as meat, vegetables and milk or other plant materials (Vescovo *et al.,* 1993). This family contains different species that display relatively high degree of diversity. Among these, *Lactobacillus plantarum* is found in many ecological niches including some dairy, meat and many traditional vegetable or plant fermentations. Furthermore *Lb. plantarum* has been attributed to probiotic activities in human and animal gastrointestine (Kalliomaki *et al*., 2001). The *Lb. plantarum* WCFS1 was the first in the genus to have the complete genome sequence determined, harbor three plasmids and serves as the model for genetic studies (Kleerebezem *et al*., 2003).

1.2 Cytoplasmic polysaccharides Glycogen

Glycogen has been determined in many bacteria as well as in eukaryotic organisms, is a polysaccharide that comprises only glucose moieties linked via the α1-4 bonds branched via the α1-6 linkages. (Fig. 4)

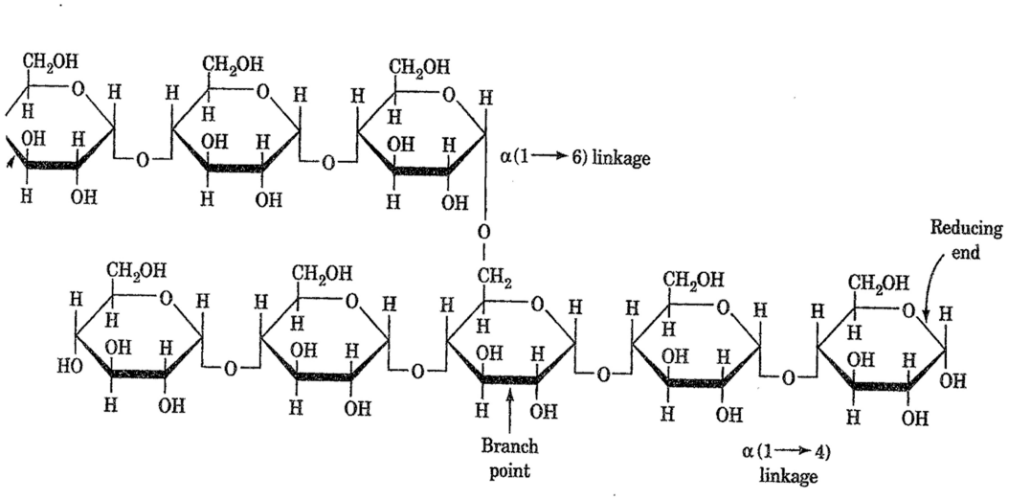


Fig. 4 schematic the glycogen structure.

The exact role of this polyglucan in prokaryotes is still unknown, although several previous studies have linked glycogen metabolism to environmental survival and even to colonization and virulence in the case of pathogenic bacteria (Bonafonta *et al*., 2000;

Bourassa, and Camili, 2009). Generally, glycogen is considered to be a storage compound providing both carbon and energy during starvation condition for the maintenance of cell integrity. During starvation or non growing conditions, bacteria need energy for growth and for several other important functions like the maintenance of mobility, intercellular pH and osmotic regulation and preservation of necessary metabolic processes by protecting some cellular constituents like RNA and their protein components from degradation to NH_3. These give the microbes an advantage to survive better with prolonged viability in the environment in contrast to those cells which do not accumulate the polysaccharide glycogen (Preiss 2003).

Glycogen biosynthesis usually occurs at the time of slow and/or no growth of the stationary phase. As the accumulation of the polysaccharide take place due to limited growth conditions like depletion of nutrients nitrogen or phosphate or even unfavorable pH, many bacterial species accumulate glycogen in the stationary phase, in the presence of an excess source of carbon.

In some exceptional cases like strains of *Streptococcus mitis* (Gibbons. 1963) and *Rhodospseudomonas capsulate* (Eidels. 1971), it has been reported that glycogen accumulates during the exponential phase of growth. However, glycogen does not seem to be essential for the growth of many bacteria like *Salmonella enterica* serovar Typhimurium*, E. coli*, *Clostridium pasteurianum, Bacillus stearothermophilus* and *Corynebacterium glutamicum* since their mutants remain unaffected in the levels of the glycogen biosynthetic growth when compared to the wild type (Preiss 1984; Takata *et al.,* 1997; Seibold *et al.,* 2007). On the other hand, in *Mycobacterium smegmatis*, recycling of the polysaccharide during exponential phase was shown to be essential for the growth (Blanger *et al*., 1999).

It has been shown that the cells of *Escherichia coli* and *Entrobacter aerogenes* as well as other Gram-negative bacterial species containing glycogen survived better than those with no glycogen and are able to preserve their protein and RNA constituents undergoing some turnover in stationary phase when present in media having no exogenous carbon source (Bornefeldt 1981; Cashel *et al*., 1974; Cattaneo *et al*., 1969). The sporulating *Bacilli* and *Clostridia* species as well as *Streptomyces coelicolor* have been shown to accumulate glycogen up to 60% of the dry weight of the organism just prior to sporulation phase and rapidly degrade at the time of spore formation. Thus, it appears that glycogen in those species serve as an endogenous source of carbon and energy for spore formation and maturation (Mackey, 1971; Slock, 1974; Chao, Weathersbee, 1974).

1.2.1 **Glycogen Metabolism**

1.2.2 ADP-glucose pyrophosphorylase

Glycogen is the major reserve of polysaccharide in bacteria. The regulation of glycogen biosynthesis from glucose 1-phosphate is achieved by controlling the expression of *glg* genes and ADP-Glc-PPase activity. This synthesis of glucose 1-phosphate is catalyzed by at least three enzymes namely ADP-Glucose pyrohposphorylase (ADP-Glc-PPase; EC 2.7.7.27), glycogen synthase (EC 2.4.1.21) and branching enzyme (EC 2.4.1.18) encoded by the *glgC*, *glgA* and *glgB* genes respectively, (Preiss. 1984, 1996). The key regulatory step of prokaryotic storage polysaccharide formation and eukaryotic photosynthetic pathway take place at the level of ADP-Glucose made via the reaction catalyzed by ADP-Glc-PPase, which forms ADP-glucose and pyrophosphate from ATP and D-glucose-1-phosphate. (Fig.5)

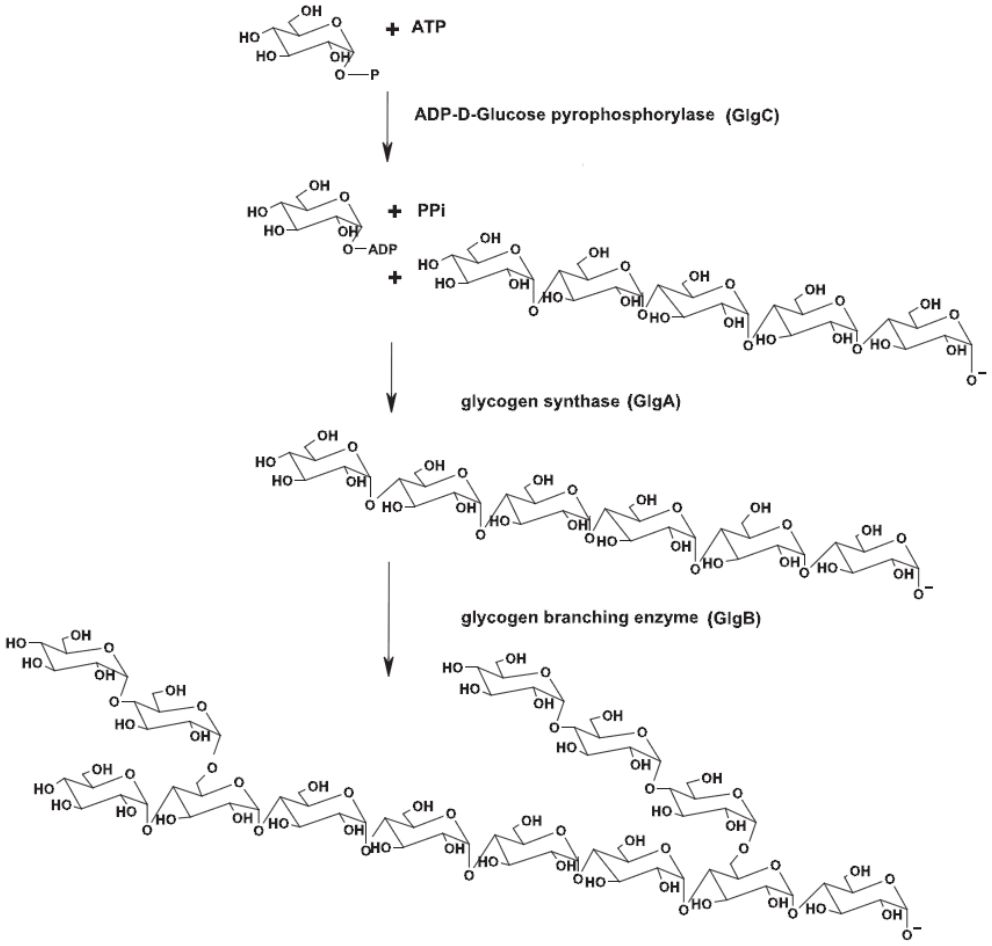


Fig.5. Schematic diagram show of glycogen synthesis in bacteria with the genes and enzyme names.

ADP-Glc-PPase (AGPPase/AGPs) has been isolated from various bacterial and plants species and all bacterial AGPs are known as a homotetrameric enzymes. Most of these

enzymes are allosterically regulated by various intermediates of the major carbon and energy metabolic pathways such as an activator by pyruvate or fructose-6-phosphate and inhabited by ADP, AMP and/or orthophosphate (Ballicora *et al*., 2003; Preiss, 1996, 1984). In the genus *Bacillus* which accumulates glycogen only during the sporulation phase, AGPPase is the only bacterial enzyme that exhibits a heterotetrameric structure of the type $\alpha_2\beta_2$. This is not affected by any substrates which act as activator or inhibitor demonstrated in other bacterial species. On the other hand, the ADP-Glc-PPase enzyme has been demonstrated in many plants and determined as the major enzyme controlling the starch synthesis. In most of the cases, the AGPPase are highly activated by 3-phosphoglycerate and (3PGA) and inhibited by orthophosphate (Pi). All of the AGPase from higher plants are a heterotetrameric structure of $\alpha_2\beta_2$ type. The subunits of the plant AGP, which are called small and large subunits (suggested as catalytic and regulatory subunit, respectively), are homologous to each other (Okita *et al*., 1990; Smith-white *et al*., 1994; Preiss. 1993, 1991).

Based on the specificity for activator and inhibitor, ADP-Glc-PPase from different prokaryotic and eukaryotic sources has been grouped into different classes as shown in Table 1(Adapted modified from Ballicoral *et al*., 2003).

Organism	Major reserve poly-glucan	ADP-Glc PPase: Allosteric regulators: Activator(s)	ADP-Glc PPase: Allosteric regulators: Inhibitor(s)	ADP-Glc PPase: Quaternary structure
Prokaryotes				
Escherichia coli	Glycogen	Fru 1,6-bisP	AMP	Homotetramer (α_4)
Salmonella enterica serovar Typhimurium				
Agrobacterium tumrfaciens *Arthrobacter viscousus*	Glycogen	Pyruvate, Fru 6-P	AMP, ADP	Homotetramer (α_4)
Rohdobacter gelatinosa *Rhodobacter glbiformis*	Glycogen	Pyruvate, Fru 6-P, Fru 1,6-bisP	AMP, Pi	Homotetramer (α_4)
Bacillus subtilis *Bacillus stearothermophilus*	Glycogen	None	None	Heterotetramer ($\alpha_2\beta_2$)
Cyanobacteria *Synechococcus* sp. strain	Glycogen	3-PGA	Pi	Homotetramer (α_4)
Eukaryotes				
Green algae *Chlorelia fusca* *Chlorelia vulgaris* *Chlamydomonas reinhardtii*	Starch	3-PGA	Pi	Heterotetramer ($\alpha_2\beta_2$)
Higher plants photosynthetic tissues Leaves of spinach, wheat	Starch	3-PGA	Pi	Heterotetramer ($\alpha_2\beta_2$)
Arabidopsis, *maize*, rice				
Nonphotosynthetic tissues Potato tubers	Starch	3-PGA	Pi	Heterotetramer ($\alpha_2\beta_2$)

Table1. Relationship between the carbon metabolism and regulatory and structural properties of ADP-Glc-PPase from different organism. The black box grouping shows the Gram-positive *Bacillus*, having different heterotetrameric structure of the ADPase enzyme compared with other prokaryotes having homotertrameric structure.
Fru, fructose; P, phosphate; bisP, bisphosphate; Pi, inorganic phosphate.

The genetic aspects of glycogen biosynthesis have been studied most comprehensively in *E. coli*. In this organism, the genes encoding the glycogen synthesis are organized in a cluster which includes the *glgC*, *glgA* and *glgB* genes. There are two more genes involved in glycogen degradation namely, *glgX* and the most distal gene, *glgP*. The latter encode glycogen phosphorylase (a member of glycosyltransferase family 35) which degrades glycogen branches by forming glucose-1-phosphate (glucose-1-P*)* (Kiel *et al*., 1994; Romeo *et al*., 1998; Preiss, 1996 and Henrissat *et al*., 2002).

Introduction

Regulation of glycogen biosynthesis in *E. coli* involves a complex and still not well defined assemblage of factors. The *glg* gene cluster in *E. coli* is composed of two tandem arranged operons (*glgCAP* and *glgBX*) and the transcription of these operons is subject to a complex regulation including catabolite repression and/or the carbon storage regulator CsrA, under PhoP-PhoQ control in the sub-millimolar Mg^{2+} concentration (Manuel *et al*., 2009; Baker *et al*., 2002). Such clustering of the genes involved in glycogen metabolism has been also described for other bacteria namely *Rhodobacter sphaeroides* and *R. capsululatus* (Igarashi and Meyer, 2000), *Mesorhizobium loti* (Lepek *et al*., 2002), *Agrobacterium tumefaciens* (Ugalde *et al*., 1998), *Corynebacterium glutamicum* (Seibold *et al*., 2007), *Bacillus subtilis* and *B. stearothermophilus* (Kiel *et al*., 1994; Takata *et al*., 1997). In the Gram-positive bacterial species, which accumulate glycogen especially in the sporulation phase, a little is known about the regulatory step. The genes for glycogen synthesis in those strains have been clustered in the operon *glgBCDAP.*

A comparative analysis of the gene clusters of the glycogen biosynthesis in prokaryotes between Gram-negative and Gram-positive bacteria shows that the regulatory step is controlled by ADP-Glc-PPase encoded by the gene *glgC* in most of the bacteria. However, in the Gram-positive *Bacillus* species, it was shown that this ADP-Glc-PPase is encoded by the genes *glgC* and *glgD* homologous to each other (Fig.6). It was indicated also that the GlgC and GlgD are the subunits of an $\alpha_2\beta_2$-type heterotetrameric enzyme, AGP-Glc-PPase which did not seem to be an allosteric enzyme in *B. stearothermophilus* (Takata *et al*., 1997).

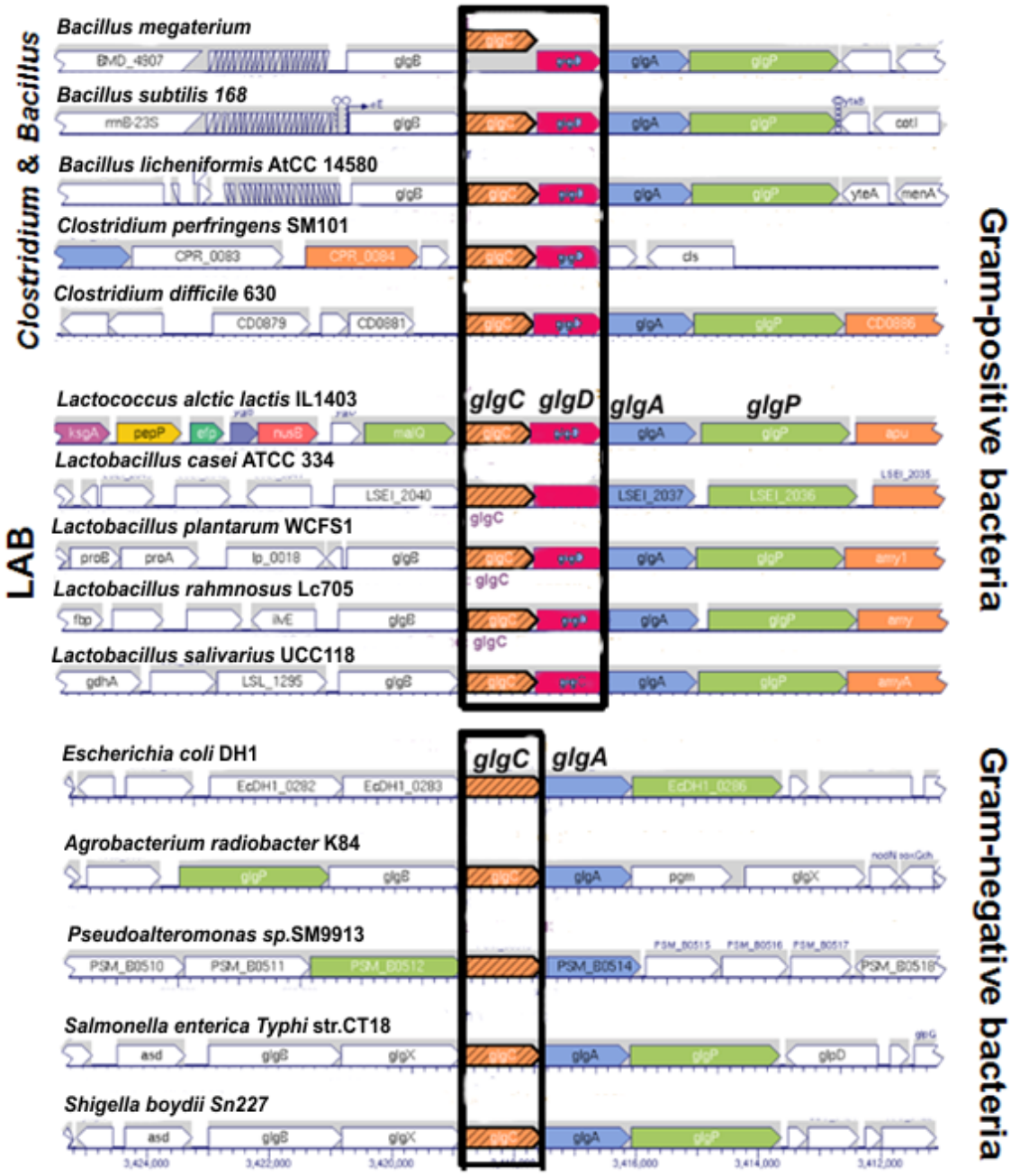


Fig.6 schematic showing the comparing the operon cluster of the glycogen biosynthesis genes in Gram-negative and positive bacteria including the sporulation genus Bacilli and clostridia and the genes cluster in some lactic acid bacteria species .

Although the GlgC protein had AGP-Glc-PPase activity without the GlgD protein, its activity was lower than that of the heterotetrameric enzyme. Again, GlgD protein did not show any AGP-Glc-PPase activity However, when the *glgC* and *glgD* genes were expressed together, the resulting GlgCD protein exhibited higher affinity for substrates and two fold higher V_{max} in catalyzing ADP-Glc synthesis than GlgC by itself. These different recombinant proteins from *B. stearothermophilus* were insensitive to the

regulation by different metabolic substrates which have been typically shown to affect the activity of other bacterial ADP-Glc PPases Thus; the enzyme is very distinct from other ADP-Glc PPases, as it is an apparently unregulated enzyme, being the only bacterial ADP-Glc PPase that exhibit a heterotetrameric structure of the type $\alpha_2\beta_2$. The GlgD protein had been assumed to be a regulator protein (Takata *et al*., 1997).

In the lactic acid bacteria, which are a group of facultative anaerobic, non-pathogenic, non-colonizing, non-sporulating Gram-positive bacterium, the genes for glycogen synthesis have been found in one or two operon cluster as *glgBCDAP* and *glgCDAP-B* in *Lb. plantarum* WCFS1 and *L. lactis* subsp. *cremoris* MG1363, respectively. The enzymatic activities of encoded protein have not been analyzed till date. Especially, the function of *glgD* gene, that is partially homologous to *glgC,* is unclear. Since *glgD* has been found only in the Gram-positive bacteria, it may be important in the glycogen metabolism of some species of Lactic acid bacteria which are recognized as probiotics.

Previous views show an important role of this storage material in serving the bacterial process. However, the precise role that glycogen might play in bacteria is still not clear.

Lactic acid bacteria are very important especially as they are related to the human health and industrial food processing.

Aim of the study

The goal of current study was to investigate the synthesis and regulation of the storage material at the level of polysaccharide metabolism in lactic acid bacteria. This would lead to the analysis of the role of the intracellular polysaccharide IPS (glycogen) as carbon and energy storage substance in LAB stains and the analysis of the functions of the biosynthetic proteins at the level of ADP-Glc synthesis, a reaction catalyzed by ADP-glucose pyrophosphorylase encoded in some of the Gram-positive bacteria including LAB by the two genes *glgC* and *glgD*.

The main flow of this work was the characterization of the enzymatic activities of the ADP-Glucose pyrophosphorylase in *Lactococcus lactis* subsp *cremoris* MG1363 and SK11 and *Lactobacillus plantarum* WCFS1: In particular, the detailed functional analysis of both the genes *glgC* and *glgD* encoding the enzyme was considered. Moreover, the function of *glgC*-homologous *glgD* being still unclear, our work aimed at the elucidation of its role in the glycogen metabolism of lactic acid bacteria.

2.1 Bacterial strains and growth conditions

2.1.1 Materials

2.1.1.1 Kits

Kit	Source
Aquapure genomic DNA Kit	Bio Rad
Biotin Chromogenic Detection Kit	Fermentas
Biotin Decalabel DNS Labeling Kit	Fermentas
Chaperone Plasmid Set	TaKaRa
Champion pET Directional TOPO Expression Kit	Invitrogen
CloneJET PCR Cloning Kit	Fermentas
Glucose detection	Megazyme
Glucose oxidaseperoxodase Kit	Sigma
Master pure DNA purification Kit	Epicenter
PureYield plasmid miniprep System	Promega
ProFound Pull-Down PolyHis Protein: Protein interaction Kit	Thermo
QIAprep Plasmid purification Kit	QIAGEN
RevertAid H minus First Strand cDNA Synthesis Kit	Fermentas
StrataClone PCR Cloning Kit	Stratagene
TOPO TA cloning Kit	Invitrogen
Wizard SV Gel and PCR clean up system	Promega

2.1.1.2 Enzymes

Enzyme	Source
Amyloglucos	Sigma
Dream Taq DNA polymerase	Fermentas
Factor Xa protease	NEB (New England Biolabs)
Glucose-6-phosphate dehydrogenase	Sigma
Klenow fragment exo-	Fermentas
KOD DNA polymerase	Novagen

Lysozyme	Fluka- Sigma
Pfu DNA polymerase	Fermentas
Phosphoglucmutase (from Rabbit Muscle)	Sigma
Phusion DNA polymerase	NEB
Proteinase K	Epicentre
Pyrophosphatase	Sigma
Quick ligase	NEB
RNase	Epicentre/ Sigma
T4 DNA ligase	Fermentas / Promega
T4 DNA polymerase	Fermentas
Taq DNA polymerase	Fermentas
UDP-glucose-pyrophosphorylase	Sigma

2.1.1.3 **Nucleic acids**

2.1.1.3.1 **Primer**

All primers used in the DNA Amplification are listed in the table.2 (overhang restriction site are underlined).

Table 2. **PCR primers**: List of the primers used to DNA synthesis by PCR. The restriction sites used for cloning purposes are underlined.

Primer	**Sequence**
mp.Rev	5´-CCGTCTAGATGGTCTGACAGTTACCAATGC-3´
Amylase.F	5´-CACCATGACCCACAAATGGTGGCAAAATGCCGC-3´
Amylase-BamHI.F	5´-CCGGATCCAGGAGAATTCAATATGACCCACAAATG-3´
Amylase.R	5´-GTTAAATACATAGACCTTACTTTCATAAGGG-3´
C2a-BamHI.F	5´-CTCGAGGATCCCGTTTCAAAGGTTATTGGAAAGATG-3´
C2-XhoI.F	5´-GGATCCTCGAGTTACTGGAATTATCGACTCCATTG-3´
C3-HindIII.R	5´-GGATCCAAGCTTCAGCATGAGAAGTCCCTTCAAACC-3´
D2-Bam.F	5´-GCGGGGATCCAAGGTCGAAAACTGTCAATTAGGTTCTG-3´
D3-Hind.R	5´-GCCTCGAGAAGCTGAGTACAGCGGTCAAATCTACATTGC-3´
ERY.F (pTRKH3)	5´-TGGGATCCGCCTGGTCGATCATTCATAAAGCAAATGCC-3´
ERY.R (pTRKH3)	5´-AGCGACTCATAGAATTATTTCCTCCCG-3´
F.Em (pMAD2)	5´-TGGGATCCGTTTATGCATCCCTTAACTTAC-3´
R.Em(pMAD2	5´CGTCTAGAACCAAATTAAAGAGGGTTATAATG-3´

F.glcA1.54-Bam	5´-CCGGATCCATTGGTGGTTAGAAAAAGGGGGGTTGC-3´
R.glcA1	5´-CGAACCCGGCTCGCTAATGGTGTTCCTTG-3´
F.CLucan-Bam	5´-CCGGATCCTGCTCAAACCGATAGTAACAAGTAAG-3´
R.Glucan	5´-GGAAAGCCTTTCTTGGCTTCCTTTTTC-3´
glg.F (MG1363)	5´-CAAGATGTTGGCATGTGGTGAG-3´
glg.R (MG1363)	5´-GAGCTGACAAACGGCTAAATCG-3´
glg-Bam.F (pWLQ-SK-C)	5´-CAGGATCCAGAAGGAATTCAGGAGCCCTTCAC-3´
glg-Sal.R (pWLQ-SK-C)	5´-GACGTCGACTGTTAGCAGCCGGATCAAACTC-3´
glg-Fusion.F	5´-TGCAGGTCGACGGATCAGAAGGAATTCAGGAGCCCTTCAC-3´
glg-Fusion.R	5´-CTTTTCCCGGGGATCCTTTGTTAGCAGCCGGATCAAAC-3
glgC-XhoI.F	5´-GATATCGAGGAGGTTCCATTATGGCAATTGAAATGC-3´
His-20.F	5´-ACGCGTACCGGTCATCATCACCATCACCATTG-3´
His-Sac.R	5´-GCCCCGCGGAGCTCTCAATGGTGATGGTGATGATG-3´
R .His.Kas	5´-GCCGGCGCCAGCTCTCAATGGTGATGGTGATGATG-3´
F.M.C-20+ up	5´-ATAAAAATGAACAATAGTGAAATGCTTGGTCTTATCCTTGCTG
MG-C+p.F	5´-TCGTCTCGAGCGGTTTGTCAGCTCATTAAG-3´
MG-C+p.R	5´-CGACTCGAGGGATGGCTGCCATTTTATTAC-3´
MG-C-his+.R	5´-GATGATGACCGGTACGCGTTTGTTCATTTTTATTTTCTCCTTTTA
MG-C_nis-Bam.F	5´-CCGGATCCGGAGGTTCCATTATGGCAATTGAAATGC-3´
F.nisA	5´-GCCCGATATGCCCAATACGCAAACCGCCTCTCCC-3´
R.nisA	5´-CGCCTCTAGACATATCCTGGTTGCAAATTTTGAAAACCGCTACG
MG-C1-Knock.F	5´-GAATTCGGAAACGGAGCGCCGTGGGGATTAAATGGG-3´
MG-C1-Knock.R	5´-GCGAATTCGACTGTTCCATCAACATAACAACCATCACC-3´
MG-D-F	5´-ATGAACAATAGTAATAAAATGGCAGCCATCC-3´
MG-D-his+.R	5´-TGATGATGACCGGTACGCGTTAAAATATCTGCAGTCACAACGG
MG-D_nis-Bam.F	5´-CCGGATCCCGGTGAAGATGAAGTCGCAGTGATTGG-3´
MG-D+p.R	5´-CTCAAGAGCGCCGATAAAGAATATCAGCGC-3´
MG-D.R	5´-CTCAAGAGCGCCGATAAAGAATATCAGCGC-3´
MG-D1-Knock.f	5´-GAATTCGGGTTCAGGAGGAGAATGGGGCTTAGATGG-3´
MG-D1-Knock.R	5´-GCGAATTCCTCGTCCATTAATGATACAACCAGAACC-3´
pHCM.F	5´-CAGGATCCTTCAGGAGCCCTTCACCATG-3´
pHCM.R	5´-TCTCTAGAACTCAATGGTGATGGTGATGATGAC-3´
pHCM4.R	5´-GGGGACGTCTCCTTTCGGGCTTTGTTAGCAG-3´
SK-C.F	5´-CACCATGGCAATTGAAATGCTTGGTCTTATC-3´
SK-C.R	5´-TTGTTCATTTTTATTTTCTCCTTTTAT-3´

SK-CD.R	5´-TAAAATATCTGCAATCACAACGG-3´
WCFS1-C.F	5´-CACCATGCAAGATGAAATGTTAGG-3´
WCFS1-C.R	5´-TGGTTGTTCCACCCCCATCGTTTCG-3´
WCFS1-D.F	5´-CACCATGAGACGTGAACAAGTG-3´
WCFS1-CD.R	5´-GTCATGCACAGCCACCTCCGCTTGTTG-3´
WCFS1-CD-mal.F	5´-CAAGATGAAATGTTAGGGATTATTTTAGC-3´
WCFS1-CD-mal.rev	5´-CAGGATCCTGGATGCTGTAGGCATAGG-3´
WCFS1-C-Knock.F2	5´-TTTCAAGGGACGGCGCACGCCATTTATC-3´
WCFS1-C-Knock.R2	5´-CGATCGCCGATATTTAACCGGTTATGGG-3´
WCFS1-D-Knock.F2	5´-GCCAGAAAAGAAGGCCGCATTACTGCAC-3´
WCFS1-D-Knock.R2	5´-CATCCAAAACGGCCATATTGGCCGCAAAG-3´

2.1.2 Strains and plasmids

The bacterial strains used in current study are listed in Tables 3 and 4. The basic and the constructed plasmid vectors are described in Table 5.

Table 3. The list of *Escherichia coli* strains used in current study.

Strain	**Description**	**Reference**
XL-1 blue	*rec*A-, *thi*, *hsd*R1, *sup*E44, *rel*A1, *lac*F', *pro*AB, *lac*Iq, *lac*ZΔM15, Tn10[Tet]	Bullock *et al*., 1987
Rosetta (DE3)	F- *omp*T *hsd*SB (*r –B m –B) gal dcm lacY*1 (DE3) pRARE6 (Cmr)	Novagen
Stbl4	*mcr*A. (*mcr*BC-*hsd*RMS-*mrr*) *rec*A1 *end*A1 *gyr*A96 *gal- thi*-1 *sup*E44 λ- *rel*A1. (*lac-pro*AB)/F. *pro*AB+ *lac*IqZ.M15 *Tn*10 (TetR)	Invitrogen
TOP10	F- *mcrA* Δ(mrr-hsdRMS-mcrBC) φ80*lac*ZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 *galE*15 *galK*16 rpsL(Str^R) *endA1* λ$^-$	Invitrogen
BL21 (DE3)	F$^–$ *ompT gal dcm lon hsdS*$_B$(r_B^- m_B^-) λ(DE3 [lacI *lac*UV5-T7 gene 1 ind1 sam7 nin5])	Novagen
XL10-Gold	endA1 glnV44 *recA1* thi-1 *gyrA96 relA1* lac Hte Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 tet^R F'[proAB l*acI* ZΔM15 Tn10(Tet Amy Cm^R)]	Stratagene
Solopack	*lac*ZΔM15, *end*A, *rec*A-,StrepR, *cre*	Stratagene, La Jolla (CA)
DH5α	F$^-$, *endA1* glnV44 thi-1 recA1 *relA1* gyrA96 *deoR nupG* Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, *hsdR17*(r_K^- m_K^+), λ–	

DH10	F^- endA1 recA1 galE15 galK16 nupG rpsL ΔlacX74,Φ80lacZΔM15.araD139,Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC) λ^-	invitrogen
JM 109	endA1 glnV44 thi-1 relA1 gyrA96 recA1 $mcrB^+$ Δ(lac-proAB) e14- [F' traD36 $proAB^+$ $lacI^q$ lacZΔM15] hsdR17($r_K^-m_K^+$)	NEB
BL21(DE3) pLysS	F–, *omp*T, *hsd*S_B (r_B–, m_B–), *dcm*, *gal*, λ(DE3), pLysS, Cm	Promega
Arctic Express	BF^- *ompT hsdS*(r_B^- m_B^-) *dcm*$^+$*Tet*^{r} *gal* λ(DE3) *endA* Hte [*cpn*10 *cpn*60 $Gent^r$	Stratagen

Table 4. The lactic acid bacteria strains including *Bacillus* and C*orynebacterium* Gram-positive strains used in this work.

Strain	Description	Reference
Lactobacillus plantarum WCFS1	*Lactobacillus plantarum* NCIMB 8826 strain WCFS1	NCIMB
Lactococcus lactis subsp. *cremoris* SK11	*Lactococcus lactis* subsp. *cremoris* NCIMB 702016 strain SK11	NCIMB
Lactococcus lactis subsp.*cremoris* MG1363	*Lactococcus lactis* subsp.*cremoris* MG1363 TMW 1.1086	Willem de Voss, Wageningen
Lactobacillus sanfranciscensis 1.54	Sourdoughs baked product	Prof. Dr. Rudi Vogel
Lactobacillus delbrueckii subsp *bulgaricus*		Göttingen- Uni
Corynebacterium glutamicum (DSM 20300T)	type strain (= ATCC 13032)	DSMZ 20300
*Corynebacterium glutamicum*R163	*C. glutamicum* ASO19 restriction deficient mutant, *tru*-	Liebl *et al.,* 1989
Bacillus subtilis 168	Our department	Göttingen-University of Göttingen

Table 5 List of basic plasmid vectors and constructs used in current study.

Plasmid	Description	Reference
pBluescript II KS	f1 (–) ori –galactosidase multiple cloning site, lac promoter, pUC ori, ampicillin resistance (bla) ORF used to disrupt the gene in lactic acid bacteria.	Short *et al*., 1988
pBlu::M-C1	Vector pBluescript KS carrying a fragment about 700 bp of gene *glg*C *L. lactis* subsp. *cremoris* MG1363	Current study

pJET1.2	High cloning plasmid rep (pMBI), *lac* promoter, *eco*47*IR*, *Amp*.	Fermentas
pET101	Expression vector in *E.coli* with P_{T7} promoter, *lac*I Amp, *His*-tag	Novagen
pET101-MG-*glg*C	Expression vector carrying the *L. lactis* subsp. *cremoris* MG1336 gene *glg*C under the control of the P_{T7} promoter	Current study
pET101-MG-*glg*CD	Expression vector carrying the *L. lactis*.MG1336 genes *glg*C and *glg*D under the control of the P_{T7} promoter	Current study
pET101-SK-*glg*C	Expression vector carrying the *L. lactis*.SK11 gene *glg*C under the control of the P_{T7} promoter	Current study
pET101-SK-*glg*CD	Expression vector carrying the *L. lactis*.SK11 genes *glg*C and *glg*D under the control of the P_{T7} promoter	Current study
pET101-WCFS1-C	Expression vector carrying the *Lb. planetarum* WCFS1 gene *glg*C under the control of the P_{T7} promoter	Current study
pET101-WCFS1-D	Expression vector carrying the *Lb. plantarum* WCFS1 gene *glg*D under the control of the P_{T7} promoter	Current study
pET101- San-α*Amy*	Expression vector carrying the ORF (*Amy*) from strain *Lb._sanfranciscensis* 1,1304 under the control of the P_{T7}	Current study
pET101-San-*gluA*$_{1.1304}$	Expression vector carrying the ORF (*αgluA*) from strain *Lb._sanfranciscensis* 1,1304 under the control of the P_{T7}	Current study
pET101-San-Glucansucrase	Expression vector carrying the ORF (*Glc*) from strain *Lb. sanfranciscensis* 1,1304 under the control of the P_{T7} promoter	Current study
pET101-WCFS1-*glgCD_his*	Expression vector carrying the *Lb. plantarum WCFS1* genes *glg*C and *glg*D under the control of the P_{T7}promoter	Current study
pK19mob*sac*B	Mobilizable *C. glutamicum* integration vector, , derivate of pK19plasmid by addition of *mob* (RP4), *sacB, Kan*^{R}	Schäfer *et al.*, 1994
pK19::ΔM-C1-Ery	Conjugated vector ,derivate of pK19mobsacB carrying *Ery* gene and 700bp of the gene MG-*glgC*	Current study
pK19::ΔM-D1-Ery	Conjugated vector ,derivate of pK19mobsacB carrying *Ery* gene and 700bp of the gene MG-*glgD*	Current study
pMAL c2x	6,6 kb,Expression vector, enables expression of proteins fused with the MBP from *E. coli*, P_{TAC}, *Male, lacZα, rrnB*, *lacI*, *Amp*	New England
pMAl c2X-W-CD	pMAL c2x expression vector carrying the genes *glg*C and *glgD* of strain *Lb. plantarum* WCFS1	Current study

pMAL3-W-C	pMAl2cx-WCFS1-glgCD_his digested with *Sac*II and *Xba*I to delete gene *glgD*$_{his}$, for interaction experiment analysis	Current study
pMAL4-W-D_his	400bp of gene glgC from construct pMAL-W-*glgCD*_his was deleted with enzyme PmlI and NcoI, used for interaction protein GlgC and GlgD analysis	Current study
pME1	Integration vector, derivate of pSP72 from Promega ,carrying Erythromycin cassette(*Em*)	Prof.vogel Labor
pME1::ΔM-*glg*C$_1$	Knockout vector carrying on fragment about 700 bp of *L. lactis* sp.MG1363 gene *glg*C (single crossover)	Current study
pME2::ΔM-*glg*C$_2$	pME1 carrying two fragment of the *L. lactis*.MG1363 *glgC* each one about 500 bp (use as a double crossover knockout vector)	Current study
pME1::ΔM-*glgD*$_1$	Knockout vector carrying on fragment about 700 bp of *L.lactis* MG1363 gene *glg*D (single crossover)	Current study
pHCMC02	ori pBR332 (for Gram-negative replication,pBS72 (for Gram-positives), MUC, *trpA* transcription terminator, weakly constitutive P $_{lepA}$ Promoter	BGSC (Schumann *et al*., 2005)
pHCMC04	ori pBR332 (for Gram-negative replication,pBS72 (for Gram-positives), MUC, *trpA* transcription terminator, P$_{xylA}$	BGSC (Schumann *et al*., 2005)
pHCMC05	ori pBR332 (for Gram-negative replication,pBS72 (for Gram-positives), MUC, *trpA* transcription terminator, *lacI* represses transcription from the P$_{spac}$ promoter	BGSC (Schumann *et al*., 2005)
pH05-WCFS1-*glg*C	Expression vector pHCMC05 carrying the *Lb. plantarum* WCFS1 gene *glg*C under the control of the P$_{spac}$	Current study
pDG148-StuI	Shuttle vector ori pBR322, ori pUB110with an adapter for cloning the PCR fragments, lac operon repressor, kan, ble, bla , under the control of the P$_{spac}$	BGSC (F.Denizot., 2001)
pWLQ2	Expression vector in *Corynebacterium glutamicum,* / *E. coli* shuttle-expression vector, *lacI*q, P*tac, rrnB, mob, oriEc* (*colE1*),*oriCg* (pSR1), ApR , KmR.	Liebl *et al.,* 1992
pWLQ-W-*glgC*$_{his}$	pWLQ2 expression vector carrying the *Lb. plantarum* WCFS1 gene *glg*C	Current study
pWLQ-W-*glgD*$_{his}$	pWLQ2 expression vector carrying the *Lb. plantarum* WCFS1 gene *glg*D	Current study
pWLQ-W-*glgCD*$_{his}$	pWLQ2 expression vector carrying the *Lb. plantarum* WCFS1 genes *glg*C and *glg*D	Current study
pMSP3535	Shuttle vector for *E. coli* and Gram-positive bacteria,*ColiE1* ori, *repD*, *repE*, *repG* have genes *NisR*, *NisK*,with *Eey*^{R}, contain a nisin-inducible promoter P$_{nisA}$	USA (Gary *et al*., 2000)

p3535-M-*glgC*	pMSP3535 expression vector carrying the gene *glg*C from *L. lactis* sp.MG1363 under the control of the promoter *nis*A	Current study
p3535-M-*glgD*	pMSP3535 expression vector carrying the gene *glg*D from *L.lactis*.MG1363 under the control of the promoter *nis*A	Current study
p3535-M-*glgCD*	Expression vector carrying the gene *glg*C and *glg*D from *L. lactis* MG1363 under the control of the nisin promoter P_{nisA}	Current study
p3535-San-α*Amy*	Expression vector carrying the ORF (*Amy*) from *Lb. sanfranciscensis* 1,1304 under the control of the promoter P_{nisA}	Current study
p3535-San-*αgluA*$_{1.1304}$	Expression vector carrying the ORF (*αgluA*) from *Lb. sanfranciscensis* 1,1304 under the control of the promoter P_{nisA}	Current study
p3535-San-*αgluA*$_{1.54}$	Expression vector carrying the ORF (*αgluA*) from *Lb. sanfranciscensis* 1,54 under the control of the P_{nisA}	Current study
p3535-San-Glucansucrase	pMSP3535 expression vector carrying the ORF (*Glc*) from *Lb. sanfranciscensis* 1,1304 under the control of the promoter *nis*A	Current study
pMSP3535H3	Improved Expression plasmid p3535H2 with *nisI* (immunity gene) and cloning reporter *LacZ* and terminator	USA (David E. Block., 2009)
pH3-M-*glgC*$_{his}$	pMSP3535H3 expression vector carrying the gene *glgC* from *L. lactis* sp.MG1363 under the control of the nisin promoter *nis*A	Current study
pH3-M-*glgD*$_{his}$	pMSP3535H3 expression vector carrying the gene *glgD* from *L.lactis* sp.MG1363 under the control of the nisin promoter *nis*A	Current study
pH3-M-*glgCD*$_{his}$	pMSP3535H3 expression vector carrying the genes *glg*C and *glgD* from *L. lactis*.MG1363 under the control of the promoter *nis*A	Current study
pH3-San-*Glu*$_{his}$	pMSP3535H3 expression vector carrying the gene Glucansucrase from *Lb. sanfranciscensis* 1,1304 under the Nisin promoter control	Current study
pMil3-MG-*glgC*$_{his}$	pMSP3535H3 expression vector carrying the genes *glg*C and *glg*D from *L.lactis*.MG1363 under the control of the promoter *nis*A, lack *LacZ* reporter	Current study
pSC-A	High copy cloning vector, *orip*UC, *lox*P, *lac* promoter, *lacZ*α, *AmpR*, *KmR* used to knockout the gene in Lactic acid bacteria	Strategen

pSC::MG-C1	Knockout vector, *orip*UC, *lox*P, *lac* Promoter, *lac*Zα, *AmpR*, *KmR* have about 700bp of the gene *glgC* from *L. lactis* MG1363	Current study
pSC::MG-D1	Knockout vector, *orip*UC, *lox*P, *lac* Promoter, *lac*Zα, *AmpR*, *KmR* have about 700bp of the gene *glgD* from *L. lactis* MG1363	Current study
pSC::WCFS1-C1	Knockout vector, *orip*UC, *lox*P, *lac* Promoter, *lac*Zα, *AmpR*, *KmR* have about 700bp of the gene *glgC* from *Lb. plantarum*WCFS1	Current study
pSC::WCFS1-D1	Knockout vector, *orip*UC, *lox*P, *lac* Promoter, *lac*Zα, *AmpR*, *KmR* have about 700bp of the gene *glgC* from *Lb. plantarum*WCFS1	Current study
pTRKH3	Shuttle cloning vector *E. coli* and Gram positive bacteria, *tet*, *ery*, AMB1 ori, p15A ori	LMBP (O´Sullivan *et al*., (1993),
pTRKH3-MG-*glgC*+p	The pTRKH3 with the gene *glgC* from *L. lactis* MG1363 with upstream region of the gene	Current study

2.1.3 Culture media and growth conditions

Growth medium for *E.coli* and *Bacillus subtilis*:

E. coli cultures were grown in Luria Bertani (LB) Medium (Sambrook *et al*., 1989). The components were added and the volume adjusted to make the complete medium which was sterilized at 120°C for 20 min in an autoclave.

LB-medium:

10 g	Tryptone
5 g	Yeast extract
5 g	NaCl
In 1000 ml	distilled water

LB solid medium: 15 g of agar was added per 1 litre of LB-medium

SOB-medium:

10 g	Peptone
2.5 g	Yeast extract
0.3 g	NaCl
0.09 g	KCl
498 ml	distilled water

After autoclaving, the filter sterilised components were added:

1 ml 1 M $MgSO_4$

1 ml 1 M $MgCl_2$ was added.

The *E. coli* cultures were grown at 37°C at 150 rpm and in some case at low temperatures between 20°C and 30°C. For the purpose of plasmid amplifications, transformed competent cells were spread on solid LB medium plates containing appropriate antibiotics to select for the plasmid and grown overnight at 37°C. Bacteria from the colonies were inoculated in 5 ml LB medium containing the same selective antibiotic as before and grown overnight with shaking at 37°C.

Substrates that are sensitive to autoclaving such as antibiotics or some sugars were sterilized by filtration (0.22 µm, Sartorius, Göttingen, Germany) and added to the media after autoclaving at a temperature between 55°and 60°C.

For blue-white screening, a dimethylformamide solution of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was added to a final concentration of 40 µg/ml. In some cases, 40 µl of 50 mg/ml stock were spread on the surface of LB-agar plates and were allowed to absorb at room temperature for 30 min.

The stock concentrations and the final concentrations of the antibiotics and other frequently-used additives in the media are shown in the Table 6

The stock solutions were sterilized by filtration and aliquoted in 1 ml volume and stored at –20°C.

Table 6 Antibiotic stock solution and final concentration used in current study.

Antibiotic	Stock solution	Final concentration
Ampicillin (Amp)	100 mg/ml in distilled water ($_{dd}H_2O$)	100 µg/ml
Polymyin B	40 mg/ml in $_{dd}H_2O$	40 µg/ml
Chloramphenicol (Cm)	34 mg/ml in 99% ethanol	34 µg/ml
Gentamycin	10 mg/ml	20 µg/ml
Kanamycin (Kan)	50 mg/ml in $_{dd}H_2O$	50 µg/ml
Tetracylin (Tet)	12.5 mg/ml in 99% EtOH	10 µg/ml.
Erythromycin (Em/Ery)	10 mg/ml in $_{dd}H_2O$ 50 mg/ml in $_{dd}H_2O$	5 µg/ml (LAB) 250µg/ml (*E. coli)*
Isopropyl-β-dthiogalacto-pyranosid (IPTG)	100 mM in $_{dd}H_2O$	0.05-0.1 mM

5-Bromo-4-chloro-3-indolyl-β-D-galactopyranosid (X-Gal)	40 mg/ml in DMF	40 µg/ml
L-arabinose (L-ara)	20% (w/v) in $_{dd}H_2O$	0.2%
Nisin	25 µg/ml in $_{dd}H_2O$	0.1-20 ng

Growth medium for Lactic acid bacteria:

L. lactis and *L. plantarum* strains were grown at 30°C in M17 and MRS medium, (Difco Laboratories) respectively.

M17 Medium for *lactococci* and *streptococci*:

Peptone from casein	5.0	g
Soya peptone	5.0	g
Yeast bacteriological	2.5	g
Ascorbic acid	0.50	g
Na2-β-glycerolphosphate	19.0	g
Lactose	5.0	g
Distilled water	up to 1000.0ml	
Adjusted pH to 6.9±0.2		

The broth was autoclaved at 121°C for 15 min.
The Lactose was sterilized by filtration and added after the medium was autoclaved.

MRS medium for *Lactobacillus* strains:

Casein peptone, tryptic digest	10.0	g
Meat extract	10.0	g
Yeast extract	5.0	g
Glucose	20.0	g
Tween 80	1.0	g
K_2HPO_4	2.0	g
$(NH_4)_2$citrate	2.0	g
Na-acetate	5.0	g
$MgSO_4.7H_2O$	0.20	g

$MnSO_4.H_2O$	0.05 g
Distilled water ($_{dd}$H2O)	up to 1000.0 ml

Adjusted pH to 6.2 to 6.5

The medium was autoclaved at 121°C for 15 min.

The glucose was sterilized by filtration and added after the medium was autoclaved.

Modified MRS for *Lactobacillus sanfranciscensis 1.54:*

Casein peptone, tryptic digest	7.0 g
Meat extract	5.0 g
Yeast extract	5.0 g
Maltose	20.0 g
Fructose	2.0 g
Tween 80	1.0 g
KH_2PO_4	2.0 g
$(NH_4)_2$citrate	2.0 g
Na-acetate	5.0 g
$MgSO_4.7H_2O$	200 mg
$MnSO_4.H_2O$	100 mg
Distilled water	up to 1000.0 ml

Adjusted pH to 5.4

Maltose and fructose were sterilized by filtration and added after the medium was autoclaved (broth medium was autoclaved at 121°C for 15 min).

Growth medium for *C. glutamicum*:

LBHIS-medium (Liebl *et al.*, 1989)

2x BHI-Medium:

Brain-Heart-Infusion (Difco)	18.5 g
tryptone	5 g
Yeast extract	2.5 g
NaCl	5 g
Distilled water ($_{dd}H_2O$)	up to 500 ml

2x Sorbitol**:**

D-Sorbitol	93 g
Distilled water ($_{dd}H_2O$)	up to 500 ml

Stock solution $MgSO_4$**:** 1 M

All above solutions were separately autoclaved at 120°C for 20 minutes and/or filtrated (0.22 µl) to sterilize. Along with 2 ml of $MgSO_4$ solution, the appropriate antibiotics were added to the medium for selection.

For solid medium: 15 g of agar was added per 1 litre of BHIS-medium.

2.2 Molecular genetics methods

2.2.1 Isolation of genomic DNA from Lactic acid bacteria

- The chromosomal preparation of genomic DNA from Lactic acid bacteria strains were isolated according to the manufacture´s manual of two commercial kits AquaPure DNA and Masterpure Gram-negative genomic DNA purification (both kits are recommended for DNA purification of Gram-negative bacteria) with following modification steps. The cell pellet of 4 ml overnight culture was resuspended in lysis buffer. The lysozyme enzyme was added to the final concentration 100 µg/ml and incubated at 37°C for 30-60 min, followed with the standard steps as described in the manual of the manufacturer´s instructions.

- Isolation of the chromosomal DNA with isopropanol/ethanol methods:

Bacterial cells were harvested from 10 ml of the overnight culture by centrifugation at 6,000xg at 4°C for 20 min. The cell pellet was resuspended gently with 300 µl of 10 mM potassium phosphate buffer pH7.5 then centrifuged as above and resuspended with 200 µl autoclaved TE buffer (50 mM Tris-HCl pH 8, 1 mM EDTA) containing 10 µg/ml of fresh lysozyme (20 mg/ml) and incubated at 37°C for 30-60 min with slight mixing every 15 min. 28 µl of 10%SDS was added and re-incubated at 37 °C for 10 min. Subsequently, 24 µl of 0.5 M EDTA pH 8.4, 20 µl of 1 M Tris-HCl pH 7.5 containing 2-5 µl RNase (100 mg/ml) was added carefully and mixed to incubate on ice for 30. To this, 10 µl of Proteinase K (2.5 mg/ml) was added directly and incubated at 37°C for 2 hr. Finally, 60 µl of the sodium perchlorate solution has been added.

The mixture was centrifuged at 13,000 rpm for 10 after adding 1 volume (Vol) of phenol/chloroform/isoamylalcohol (PCI) (25:24:1).
Carefully the supernatant (water-phase/upper phase) was transferred into a new clean 1.5 ml microfuge tube. In case of improper phase separation, the centrifugation step was repeated for 5 min). 1 volume from chloroform/isoamylalcohol (24:1) was added; the sample was mixed and centrifuged at the maximal speed at 4°C for 10-15 min. The clear supernatant (upper water phase) was transferred further into a new clean 15 ml falcon tube. The DNA precipitation was carried out by adding 1.Vol of isopropanol and inverting the tube 30-40 times before centrifugation at high speed of 13,000 rpm at 4°C for 10 min. The supernatant was carefully decanted without disturbing the pellet. The DNA pellet was washed twice with 70% ethanol at room temperature. Finally, the vacuum or air dried pellet was eluted with TE buffer pH 8 or autoclaved Milli-Q water.
The genomic DNA samples were stored at -20°C until used for further experiments.

2.2.2 PCR amplification

The PCR process carried out in an automated thermal cycler (My Cycler, Bio Rad) usually consisted of 30-35 cycles and comprises three major steps: DNA denaturation, primer annealing and primer extension. For expression purposes the target genes were amplified using an enzyme having proofreading activity like *Pfu*, KOD HiFi and phusion polymerase (having high fidelity and lower PCR mutation frequency than *Pfu* polymerase enzyme), otherwise Dream *Taq* was used.
The following general PCR mixture components were used for amplification of the target DNA using the plasmid or genomic DNA as the reaction template:

Table 7a. Components PCR of amplification mixture using *Pfu* polymerase

Reaction mix	**Final concentration**	**Volume**
DNA template	50-1 µg	1 µl
Forward primer	0.1-1 µM	2 µl
Reverse primer	0.1-1 µM	2 µl
dNTPmix (each 2mM)	0.2mM	2 µl
Pfu polymerase	1.25-2 U	0.2 µl
10x buffer + $MgSO_4$	1x	2µl
Water nuclease free (dd H_2O)	Up to 20 µl	10.8 µl

Table 7b. Components of PCR amplification mixture using *KOD* polymerase

Component	Final concentration
10 x Buffer	1 x
25mM MgCl2	1,5 mM
dNTPs (2 mM each)	0.2 mM (each)
Water nuclease free	Up to final volume
Forward Primer	0.4 µM
Reverse Primer	0.4 µM
Template DNA	0.006-6 ng (plasmid) Up to 12 ng (genomic DNA)
KOD polymerase	0.02U/µl

The standard PCR parameters were used (Tab.8):

Table 8. The standard cycling parameters used in current study

Step	Temperature	time	
Initial denaturation	95-98°C	2-3 min	
Denaturation	95-98°C	15-30 sec	30-35x cycle
Annealing *	$T_A=T_m$ -5°C	15-30 min	
Extension**	72°C	2min/kb**	
Final extension	72°C	5-10 min	
store	4-8°C	forever	

* T_A optimal annealing temperature. For primers containing less than 25 nucleotides, the approx. melting temperature (Tm) can be calculated using the following equation: $Tm = 4 (N_G + N_C) + 2 (N_A + N_T)$, where G, C, A, T represent the number of respective nucleotides in the primer.

** Because the KOD HiFi polymerase has a high processivity, a long extension time may cause smearing. The elongation time is just 30-60s in total.

2.2.3 Dephosphorylation of linearized plasmid DNA

In order to reduce self ligation frequency of the linearized plasmid DNA during ligation reaction, the removal of the 5´ phosphate group was catalyzed from the end of the linearized vector by alkaline phosphatase treatment with the enzyme Antarctic Phosphatase–AP (New England Biolabs, Frankfurt, Germany). The reaction was performed according to the protocol provided by the manufacturer.

2.2.4 Overlap extension PCR(Fusion PCR)

The first step corresponds to a conventional PCR amplification. The reserve primers R-M-C_$_{his+}$, R-M-D_$_{his+}$ referred to as P1 or P2b respectively, of the fragment 1 is complementary at 5´end of the forward primer F-his+ (P3) of fragment 2 (which amplified using the previously primer as shown in Fig. 7). This complementary region is necessary for the fusion of the two fragments 1 and 2 in the second PCR step. The homologous flanking region was about 20 bp long followed by 21 nucleotides perfectly matching to the sequence of the fragment that has been amplified. Standard optimal PCR program was used to amplify the fragments 1 and 2, the PCR product from fragment 1 was purified from the agarose gel and mixed with the fragment 2 under the following PCR program that was used to amplify the final fusion PCR without primers:

Step	Temperature	time
Denaturation	95-C	2 min
Annealing	$T_A=T_m$ -3°C	1 min
Elongation	72°C	10 min

Cycle No. 10 or 30

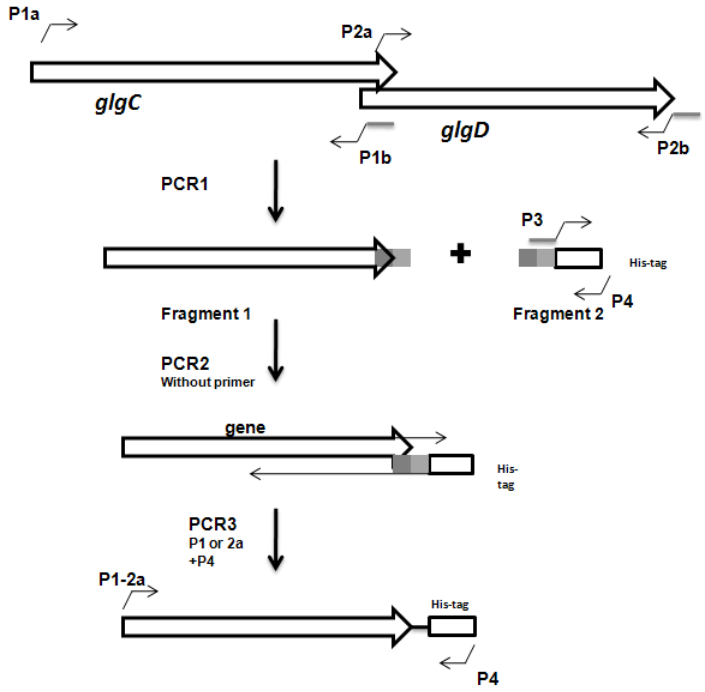


Fig.7. Schematic diagram showing the overlapping extension PCR strategy used to create a fusion target with the His-Tag downstream of the target gene. The His-tag was obtained from the plasmid vector pET101 for facilitating the purification of the expressed gene.

Subsequently, both the forward P1-2a and the reserve primer P4 were combined to the mixture for the optimal standard PCR cycler program. The fusion PCR product was separated on 0.8% agarose gel. Further, the correct band size was purified from the agarose gel as recommended in the protocol of the manufacturer´s instructions (Promega).

2.2.5 Generation of blunt DNA ends

As a prerequisite of some cloning reaction, blunting of the 3' and 5' ends of the DNA fragments were done by incubation with T4 DNA polymerase. The T4 DNA polymerase lacks 5´→3´ exonuclease activity but has a 3´→ 5´ exonuclease activity which could be used to fill in the 5´ends of the product with dNTPs, but not for the generation of blunt ends at the 3´overhang.

The reaction was carried out at room temperature for 5 min.

5X reaction buffer	4 µl
Linear DNA Fragment/ PCR product	1 µg
dNTP mix each 2mM	1 µl
T4 DNA polymerase	1 U (0.2 µl)

Nuclease free water	up to 20 µl

The reaction was stopped by heating at 75°C for 10 min.

2.2.6 Attachment of Adenosine 3´overhangs:

An A´overhang is needed in some cloning cases as in the plasmid vector pSC-A, especially if the PCR product had been amplified with proofreading DNA polymerase like *Pfu* or KOD. To obtain the modified T/A end, the purified PCR product was treated with thermostable *Taq* DNA polymerase enzyme.
The reaction was carried out at 72°C for 1 hour and the protocol for the mixture is shown below:

PCR product		1 µg
Taq-buffer+ $(NH_4)_2SO_4$ (10x)		1 x
dATP (10 mM)		1 mM
Taq-DNA polymerase		1 U
Nuclease free water	up to	30 µl

2.2.7 Ligation of DNA fragments

The ligation reaction was used to insert DNA fragment into plasmids. T4 DNA ligase (Fermentas/Promega) was employed to establish covalent phosphodiester bonds between 5´phosphate and 3´hydroxyl ends of the double stranded DNA fragment with blunt end or cohesive-end termini.
DNA fragments were mixed in the molecular ratio of 1:3, 1:4 and1:6 of vector (50-100 ng) to the insert, respectively. Ligations were performed in a total volume of 20 µl. The following formula was used in order to determine the vector/insert ratio of required for ligation:

Vector [ng] / vector size [bp] = Insert [ng] / Insert size [bp]

The Ligation reaction:

50-100 ng	vector
Variable (X mole)	insert
1X T4-ligase	buffer
5-10 Units	T4 ligase
Up to 20 µl	distilled water

The reaction was performed at RT for 30 min to 3 h or at 16°C overnight.
The following procedure was used for the Quick ligation reaction (NEB , Ipswich, MA, USA):

50 ng	Linearized plasmid DNA
X mole	purified insert
1 x	2x Quick ligase buffer mix
1 U	Quick T4 ligase
Up to 20 µl	Nuclease free water

(The reaction was mixed, centrifuged briefly 1-2 sec and incubated at RT for 5 to 30 min).
Afterwards, the ligase enzyme was inactivated at 70°C for 5 min. In case of electro-transformation, the ligation mixture was purified using Wizard SV Gel and PCR clean up system (Promega). SureClean solution (Bioline, Luckenwalde) give a high recovery of DNA than from the column purification method, (according to the the manufactures' manual).

2.2.8 Linker ligation

To introduce a small DNA fragment (His-Tag DNA sequence of pET101) into the target gene, a modified ligation procedure was performed. The DNA primers were designed to be complimentary to each other for about 20-30 bases having the 5´ end for the forward primer homologous to the target gene sequence and the restriction enzyme site linker for the reverse one. About 10 µM of each primer was mixed with sterile water and heated at 95°C for 5-10 min. The mixture was cooled down slowly at RT to anneal the homologous fragments and then incubated on ice. The resulting double strands were used further in fusion PCR reaction with the target gene.

2.2.9 Gene Cloning

<u>TOPO cloning</u>
The overexpression system pET101(C-terminal, with V5.6xHis, fusion tag and selection marker Ampicillin) was used according to the manufacturer's instructions (Invitrogen)

T/A-Strata Clone PCR Cloning

This strategy was used to create a knockout vector by replacing a part of the coding region of the target gene via homologous recombination. This system allows direct selection of recombinants. The cloning was performed as described in the kit manual (StrataClone PCR Cloning Kit, Stratagene). An additional erythromycin resistance cassette was amplified from plasmid pMAD2 as a special selective marker for Lactic acid bacteria by using the Em primers introduced by restriction enzyme sites *Bam*HI and *Xba*I for facilitating the cloning procedures. The purified PCR product was digested with the enzymes and cloned into the knockout vector pSC-Δtarget-*glg* gene at the same enzyme sites. In the resulting single-crossover, integration vectors pSC-Δ*glg* gene::*em* were used for further gene knocking out experiments.

Ligation Independent Cloning (LIC)

Ligation independent cloning (LIC) was developed for PCR products without using restriction enzyme digestion and ligation reaction (Aslanidis. *et al*., 1990; Haun *et al*., 1992) with the advantage of T4 DNA polymerase to generate long sticky ends of 12-15 nucleotides. The LIC vector, which has a special adapter, is created by treating the linearized backbone with T4 DNA polymerase in the presence of only one dNTP. The 3´to 5´exonuclease activity of the T4 DNA polymerase removes nucleotides until it encounters a residue corresponding to the single dNTP (dTTP in our case) present in the reaction mixture. The insert is amplified by PCR with the overhang of about 12-15 nucleotides having the indicated complementary sequence of the vector´s adapter. This cloning strategy for the target genes into *Bacillus* expression vector pDG148 was previously described by Joseph *et al*. (2001).

CloneJET1.2 PCR Cloning

The high efficiency cloning system pJET1.2 blunt vector contains a gene for the lethal restriction endonuclease *Eco*32I, which is interrupted by ligation of a DNA fragment into the cloning site of the vector. Therefore, only recombinant clones carrying the plasmid with insert were grown on selective medium, while the re-circularized vector lacking an insert will express a gene lethal to the host *E. coli* cells. The ligation reaction was carried out according to the manufacturer´s instruction. All needed reagents and

instruction were provided with the CloneJET PCR cloning kit (Fermentas, Vilnius, Lithuania).

2.3 Transformation

2.3.1 Preparation of high efficiency electro-competent *E. coli* cells

Electroporation provides a method of *E. coli* transformation with efficiencies greater than are possible with the best chemical methods. The survival and transformation of cells is related to intensity of the field (field strength=voltage/distance between electrodes) and to the length of the pulse (time constant).
The procedure used for the preparation of the competent cells is described as following:

1- A fresh *E. coli* overnight culture was inoculated in 1 L of autoclaved YENB (0.75 % Bacto yeast extract, 0.80% Bacto Nutrient Broth) in 2 litter flask.
2- The cells were grown at 37°C with vigorous shaking to an approximately OD_{600} of 0.5-0.7 (the best results are obtained with cells that are harvested at early-to mid-log phase; the appropriate cell density therefore depends on the strain).
3- The cells were chilled on ice for about 5 min and spun down in cold centrifuge at 4°C with speed maximal at 4,000-5,000 xg for 10 min.
4- The supernatant was removed and the pellet gently re-suspended and washed first time with 200 ml cold water and centrifuged as described above.
5- The cells were re-washed with 50 ml of ice cold 10% glycerol and cells were spun down by centrifugation as mentioned above.
6- The cells were re-suspended in 2-3 ml of ice cold 10% glycerol and aliquoted to 50-100 µl in cold 1.5 µl clean tubes and immediately frozen in liquid nitrogen.
7- Aliquoted cells were stored at -70°C (for 1-2 years)

2.3.2 Preparation of chemical competent *E.coli* cells(Inoue et al.,1990)

This protocol differs from other procedures in that the bacterial culture is grown at temperature lower than the conventional temperature of 37°C (it is thought that perhaps the physical or composition characteristics of the *E. coli* membranes which synthesized at low temperature about 19°C may be have higher favourable for DNA

uptake, or the growth phases could be extended of the efficient transformation).

1- A single colony of *E. coli* strain was used to inoculate 5 ml of SOB medium for overnight growth.
2- The overnight culture was used to inoculate 250 ml of SOB-medium and incubated at 19°C with vigorous shaking at 200-225 rpm in a baffle Erlenmeyer flask to OD_{600} of about 0.5-0.6 which normally takes 24-36 hours depending on the *E. coli* strain.
3- The culture was then transferred to an ice-water bath for 10 min.
4- The cells were harvested by centrifugation at 4,000 rpm at 4°C for 10 min.
5- The cells were gently resuspended in 80 ml ice-cold TB buffer swirling and stored on ice again for 10 min.
6- The cells were harvested again as described above.
7- Finally, the cell pellets were gently re-suspended in 20 ml ice cold TB and 1.4 ml DMSO was added (it is important the DMSO be stored at -20°C before use).
8- The bacterial suspension was mixed by swirling and then quickly the re-suspended cells were aliquoted to100 µl in precooled 1.5 ml microfuge tubes and immediately frozen by immersing the tightly closed tubes in a bath of liquid nitrogen.
9- The tubes were stored at -70°C until needed. For most cloning purposes 50 µl aliquots will be enough. This competent *E. coli* cells were transformed by the heat shock method.

SOB solution:

0.5% yeast extract
2% tryptone
10mM NaCl
2.5mM KCl
10mM $MgCl_2$
10mM $MgSO_4$
Dissolved in nanopure water and autoclaved to sterilize.

TB solution:

10 mM PIPES
15 mM $CaCl_2$
250 mM KCl

Dissolved in milliQ water and adjusted the pH to 6.7 with KOH or HCl followed by the addition of $MnCl_2$ to a final concentration of 55 mM, and adjustment to the final volume.

Sterilization was done by filtration with 0.45 µm filter and samples were stored at 4 °C.

2.3.3 **Gene transfer to *E. coli***

Heat shock:

Competent cells were thawed on ice and 0.1-10 µg DNA vector was added. The suspension was mixed manually by hand without using the pipette and further incubated on ice for 20-30 min. The suspension was exposed to heat shock in water bath at 42°C for 30 to 50 sec depending on the *E. coli* strain. Afterwards, the samples were incubated for 3-5 min on ice to reduce damage to the cells. A 900 µl pre-warmed at RT LB/SOB medium (without antibiotic) was added and the culture was incubated at 37°C for 1 hr with shaking. Different dilution of the resulting cultures 50, 100, 200 µl and the rest were spread on selective LB-agar plates and grown at 37°C overnight.

Electroporation:

To transfer the DNA into *E. coli* cells by electroporation, the electro-competent cells (preparation was described above section 2.3.1) were thawed on ice and mixed by flicking the tube with 1 µl DNA to give a final concentration (10 pg-100 ng). Care was taken to get pure DNA free of all salts. The desalting and purification of the DNA vector was carried out on Millipore membrane in MilliQ water for 20-30 min or by using SureClean solution. In case of transforming the DNA ligation mixture, before transferring into a cold electroporation cuvette 2 Cm (PeqLab, Erlangen, Germany), care was taken to remove the ligation salt buffer and ligase enzyme which might be inhibiting the transformation efficiency. A short electric pulse was applied on the Gene Pulser II chamber (Bio-Rad, Munich, Germany), with the parameters of 2.5 kv, 25 µF and 200 Ohm resistance to give a time constant between 4.5-5 ms. The transformation cell mixtures were immediately mixed with the pre-warmed S.O.C or LB medium and incubated at 37°C with shaking at 225 rpm for 1 h. At the end of recovery period, the cells were plated in different dilution on a selective LB-Agar medium to screen for transformants.

2.3.4 **Preparation of *Lactococcus* and *Lactobacillus* electro-competent cells**

The electrocompetent cells of *Lb. plantarum* WCFS1 were prepared by different methods as follows: Freshly electro-competent cells were prepared as described by Teresa Alegre *et al*., 2004, Auskrust 1995, Natori *et al*., 1990 and Wei *et al*., 1995. Frozen competent cells were prepared as described by Françoise Bringel and Jean-Claude Hubert (1990).

L. lactis electro-competent cells were prepared by modifying the method of (Maria *et al*., 2007). An overnight culture of *L. lactis* inoculated in GM17 (M17 medium supplemented with 1.5% glucose) broth containing 1% glycine and supplemented with 40 mmol/l threonine (Sigma), was diluted in 250ml SGM17 (GM17+0.5 M sucrose). The cells were harvested by cold centrifugation at 8,000xg for 10 min when the optical density at 600 nm was between 0.3-0.4 according to Dorman and Collins (1990). The cells were suspended and incubated at room temperature for 30 min in 80 ml of 100 mM LiAc (Lithium acetate), 10 mM DTT (dithiothreitiol), 0.6 M sucrose and 10 mM Tris-HCl pH 7.5. The cells were pelleted as described above.
The pellet was re-suspended gently with 250 ml of ice cold electroporation buffer without EDTA and centrifuged as before. The pellet was re-suspended with 125 ml of ice cold electroporation buffer with EDTA and chilled on ice for 15 min followed by centrifugation at 4°C. The cells were washed for the third time with 250 ml of ice cold electroporation buffer without EDTA. Finally, the cell pellets were re-suspended in 2 ml of ice cold electroporation buffer and frozen into aliquots of 50 to 100 µl in liquid nitrogen and stored at -80°C. These electrocompetent cells are stable for at least six months.

2.3.5 **Gene transfer to lactic acid bacteria strain by electroporation**

The electro-competent cells were freshly used or thawed on ice for maximal 10 min and transferred into a chilled sterile electroporation cuvette (PeqLab, Erlangen, Germany).1-2 µl of purified DNA was added and all air bubbles before electroporation were removed. The electroporation procedure carried out at 1.5 kV, 25µF and 300 ohm in a GenePulser II chamber (Bio-Rad, Munich, Germany) with a pulse duration between 4.5 and 5 ms. Subsequently, 900µl of the ice-cold MRS or GM17 was added and incubated the transforming mixture on ice for 10 min. The transformation mixture

was further incubated at 30°C without shaking for 2 and 3 h for *Lactococcus* and *Lactobacillus* strains, respectively. Finally, different aliquots of transformed cells were spread on MRS/M17plate supplied with 5 µg/ml erythromycin and were incubated at 30 °C for 2 days.

2.3.6 **Preparation of the *Bacillus subtilis* natural competent cells**

Competence cells are in a certain physiological state in which the cell is able to bind and take up the DNA. The natural competence in *B. subtilis* develops during the transition from the exponential to the stationary phase of the growth.
The procedure used in this work described by Kunst and G. Rapoport (1995) is an efficient method of obtaining high yield of the natural competent cells.
A heavy streak of the *B. subtilis* 168 glycerol stock was plated on LB agar and incubated at 37°C overnight. The following day, one colony was scraped off and inoculated in 5 ml of pre-warmed LB medium and incubated at 37°C overnight under vigorous aeration. The day after, 10 ml of the medium MNGE supplemented with CAA was inoculated with overnight culture to obtain an OD_{600} of less than 0.1 and incubated until the beginning of the stationary phase at about OD_{600}= 1.2-1.3. Thereafter, the culture was supplemented with freshly prepared 10 ml MNGE medium without CAA, and incubated at 37°C for an additional hour. The cells were accumulated by centrifugation at 8,000xg at RT for 5 min. The pelleted cells could be used directly for transformation by re-suspending in a minimum volume of the supernatant as freshly competent cells. They could be also frozen at -80°C after re-suspending the pellet with 1/8 volume of the supernatant by adding sterilized glycerol solution to final concentration 10% and aliquoted in 400- 500 µl.

10x MN-medium:

K_2HPO_4(3x H2O)	136 g
KH_2PO_4	60 g
Sodium citrate	10 g

MNGE- medium:

1x MN-medium	9 ml
Glucose (20%)	1 ml
$MgSO_4$ (1M)	30 µl
Tryptophan (5mg/ml)	100 µl

Ammonium citrate (2.2 mg/ml)	50 µl
Potassium glutamate (40%)	50 µl
±10% Casein hydrolysed (CAA)	100 µl

2.3.7 **Gene transfer to *Bacillus subtilis* 168**

1-2 µg of purified DNA was mixed with 400 microlitres of competent cells (freshly or thawed on ice) and was incubated with shaking at 37°C for 30 min. Subsequently, 100 µl of the expression solution was added and the mixture was incubated at 37 °C additionally for one hour with shaking at 150-160 rpm. Finally the cell suspensions were spread onto LB-agar plates containing the appropriate antibiotics for selection (5 µg/ml chloramphenicol).

Expression solution:

Yeast extract (5%)	500 µl
CAA (10%)	250 µl
$_{dd}H_2O$	250 µl
Tryptophan (5mg/ml)	50 µl

2.3.8 **Preparation of the *C. glutamicum* R163 competent cells**

Preparation of the competent cells

10% glycerol solution: the solution was sterilized by autoclaving and stored at 4 °C.

Epo additive	soniazide	400 mg
	Glycine	2.5 g
	Tween80	100 µl
	$_{dd}H_2O$	20 ml

The above solution was freshly prepared

The procedure for the electrocompetent cells preparation was described by Van der Rest *et al*., 1999.

2.3.9 **Gene Transfer to *C. glutamicum***

Electrotransformation:

To introduce the heterologous DNA into *rec*$^+$ *C. glutamicum* strains, the first step was to electroporate the DNA into R163 cells, followed by the re-purification of the plasmid and finally electroporation into the *rec*$^+$ strains (Liebl *et al*., 1989a). The transformation procedure is the same for both the strains.
The competent cells were thawed on ice for 5-10 min and transferred into a chilled sterile electroporation 2 mm cuvette (PeqLab, Erlangen, Germany). 1-2 µl of purified DNA from R163 strain was added (100-200 ng) and all air bubbles were removed before electroporation. The procedure was carried out at 2.5 kV, 25 µF and 200 Ω in a GenePulser II chamber and pulse duration between 4-5 ms was applied. Subsequently, 1 ml of the pre-warmed BHIS was added .The transformation mixture was incubated at 30°C with shaking at 150 rpm for 1 hr; finally, different aliquots of transformed cells were spread on selective plates and incubated at optimal temperature overnight.

2.4 Plasmid extraction

2.4.1 **Isolation of the plasmid DNA from *E. coli***

2.4.1.1 Plasmid preparation by alkaline lysis (Birnboim and Doly, 1979)

The isolation of plasmid DNA described by Birnboim and Doly (1979) is based on the different behaviour of chromosomal and plasmid DNA at pH values from 12 to 12.5. While the chromosomal DNA could be denatured and pelleted under these conditions and removed by neutralization step, the supercoiled plasmid DNA remains still in the solution and will be accumulated by precipitation with isopropanol or by binding to a silica-membrane. For this propose, 5 ml of E. coli culture with the selective antibiotic of the specific plasmid was incubated at 37° C overnight. Two to five millilitres of the overnight culture were transferred to a sterile 2 ml microcentrifuge tube and then harvested by centrifugation (13,000 rpm, at 4° C for 2 min, MiniSpin plus, Eppendorf Hamburg). The cell pellet was completely resuspended in 200 µl buffer 1 (P1, plus RNase A). After addition of 200 µl buffer 2 (P2), the mixture was gently inverted several times. For the precipitation of proteins and chromosomal DNA the reaction mixture was

neutralized with 200 µl buffer 3 (P3). Following this, the sample was centrifuged at 13,000 rpm at 4°C for 20-15 min; the supernatant was transferred into a fresh 1.5 ml microcentrifuge tube and mixed with 500 µl isopropanol (AppliChem GmbH, Darmstad, Germany). The mixture was incubated on ice for five minutes and the plasmid DNA was pelleted by centrifugation at 13,000 rpm, at 4° C for 20 min. The pellet was finally washed twice with 800 µl 75% ethanol (J.T. Baker, Deventer, Holland), and dried under the air or in the vacuum centrifuge (Speed Vac plus SC110A, Savant/Göttingen). At the end, the plasmid DNA pellet was resuspended in 30 µl sterile H_2O_{bidest}.

Resuspension buffer (P1):

10 mM EDTA (MG 372.2)		0.37 g
50 mM Tris-HCl (M G121.1)		0.605 g
$_{dd}H_2O$.	up to	100 ml
pH 8.0		

The buffer was sterilized by autoclaving at 120°C for 20 min, then RNase stock solution was added 0.1 % (v/v) and stored at 4°C.

Lysis buffer (P2):

0,2 M NaOH (MG 40.0)		0.8 g
1%SDS		1.0 g
$_{dd}H_2O$	up to	100 ml
Stored at RT		

Buffer (P3- Neutralization and precipitation):

3,2M otassium acetate (MG 98.15)		31.36 g
$_{dd}H_2O$	up to	100 ml
pH 5.5 (adjusted with glacial acetic acid. 25ml)		
Stored at RT		

RNase stock solution (50x):

RNase A	10 mg/ml
Tris	50 mM
$Na_2EDTA{\cdot}2H_2O$	10 mM
pH 8.0	

The RNase stock solution was boiled for 15 min in a water bath to inactivate DNase.

The solution was then aliquoted into 1.5 ml microcentrifuge tube and stored at -20 °C until used. The solution was added to the autoclaved buffer immediately before use.

2.4.1.2 Colony cracking

A small quantity of the bacterial colony was removed from the selective plate using a sterile toothpick and the cells well re-suspended in 1.5 ml clean tube with 15 µl suspension buffer (10 mM EDTA pH 8). To this mixture, 15 µl of fresh lysis buffer P2 was added (P2: 0.2 M NaOH, 10% SDS or 5 M NaOH+10%SDS and 0.2 g sucrose in 1 ml of water) to ensure that the content are well mixed and then the mixture was incubated at 95°C for 10 min. This incubation was followed by cooling down the samples to room temperature. Loading buffer (1.5 µl of 4 M KCl+1 µl loading dye prepared as master mix) was added, mixed well and then chilled on ice for 10-15 min to precipitate out the proteins and then centrifuged at maximal speed of 13,000 rpm at 4°C for 10 min. Relatively large volume of the supernatant was loaded on the agarose gel to ease visualization of the DNA taking into account that the quantity of the DNA from a single colony is small. The size of the recombinant plasmid in each lane was compared with the empty plasmid as control.

2.4.1.3 Plasmid purification by QIAGEN mini prep Kit

2-4 ml of the 5 ml overnight *E. coli* culture harbouring the plasmid constructs were pelleted in 2 ml reaction tubes by spinning down twice 2 ml of the cells for 2-3 min at maximal speed. The cell pellet was then treated as described in the manual of the manufacturer´s instructions QIAprep spin Miniprep kit (QIAGEN, Hilden, Germany).

2.4.2 **Plasmid isolation from *C. glutamicum***

Presence of the thick layer of peptidoglycan in Gram-positive bacteria requires additional cell-wall degradation. Therefore, cells from of the 5 ml overnight LBHIS culture of *C. glutamicum* R163 were harvested at maximal speed of centrifugation for 5 min and then the pellet was resuspended in 80 µl TE buffer containing freshly prepared lysozyme (20mg/ml) solution and incubated at 37°C for 1 hr. The degradation reaction was stopped by adding 10 µl of 0.5 M EDTA or by adding the suspension buffer P1. The following next steps were done as described early in section 2.4.1.1.

2.4.3 **Plasmid extraction from lactic acid bacteria**

A modified method was used for plasmid purification from *L. lactis* subsp. *cremoris* MG1363.
The protocol of QIAGen Miniprep plasmid purification has been modified by adding lysozyme to 5 ml of the suspension of cells pellet after harvesting and incubated at 37 °C for at least 1 hr with short and quick ultra-sonification treatments of 5-10x for 1 s helping sometime. Subsequently, plasmid extraction was continued as described in the manual of the manufacturer´s instruction manual of QIAGen miniprep kit. Small amount will be further purified from the plasmid 50-80 ng/µl according to this method. The low plasmid concentration could not be visualized on the gels. Thus, the same construct was re-transformed into *E. coli* XL1/XL10 blue to get huge amounts of plasmids which were isolated for further experiments.

2.4.4 **Determination of the DNA concentration**

The concentration and the purity of the DNA were determined by using Nano-Drop spectrophotometer ND-1000 from Peqlab Biotechnology (Erlangen, Germany).
DNA concentrations are determined by measuring the absorbance of the sample at wavelength 260 nm. However, the disadvantage of this method is poor sensitivity and impossibility to distinguish between single-stranded and double-stranded DNA. The purity of the DNA could be realized by ratio OD_{260}/OD_{280}.
The alternative method to estimate the DNA amount was comparison of the intensity of the bands after agarose gel separation with suitable DNA ladder bands of comparable size and defined concentration.

2.4.5 **Restriction analysis**

For restriction analysis, 1-4 µl of the plasmid corresponding to approximately 0.2-0.5 µg of DNA was used. 1 µl of the 10X recommended buffer, 1 µl of 10X BSA (if necessary), at least 1-2 units of the adequate restriction enzyme (0.5-1 µl) and distilled water were added to the final reaction volume of 10 µl. Finally, the reaction mixture was incubated for 1-2 h at the recommended enzyme temperature.
For preparative digestion, 1-3 µg of plasmid DNA was used out in a total reaction

volume of 40 µl at the recommended enzyme temperature for at least 4 h until overnight.

2.4.6 **Agarose gel electrophoresis**

Digestion of the DNA fragments were analysed or purified by gel electrophoresis. Digested plasmid DNA was separated in a 0.8-2% (w/v) agarose gel according to the expected size of the DNA fragments. All samples were mixed with 0.20 volume of a 6x DNA loading dye (Fermentas) and run in 1X TAE buffer at a constant voltage of 110-120V (BioRad Power Pac 300 power supply) for 40 min. The DNA gel was stained in ethidium bromide solution bath (1.5 µg/ml EtBr solved in water) for 5-10 min, followed by washing with ddH_2O. The stained DNA bands were visualized by UV light and documented using a GelDoc system Alpha Innotech. Subsequently, the stained gel was photographed using Alpha imager software. The DNA ladder (GeneRuler 1 Kb/1 Kb plus/50bp DNA Ladder, Fermentas) was separated on the same gel in order to estimate the DNA fragment size or approximately determine the DNA concentration of analyzed DNA

The running buffer TAE was made into a 50x solution, which was diluted to 1X before use

Tris-acetate- EDTA (TAE) buffer 50X

Tris base		242g
Acetic acid		57ml
0.5 M EDTA (pH 8.0)		100ml
$_{dd}H_2O$	add	1000ml

6x DNA loading buffer

Glycerol	30 ml
Tris-HCl (150mM pH7.6)	50mM
Bromophenol blue	125mg
$_{dd}H_2O$	3.5 ml

2.4.7 **Colony PCR to identify the positive colony**

The colony PCR was used for quickly screening a large number of the recombinant *E. coli* at the same time. A small portion of the well-grown colonies were picked with a

sterile toothpick and re-suspended in PCR tube containing 10 µl of ddH_2O. The PCR was performed by adding mixture of the components to the re-suspended cells (The primers can bind to the genomic DNA of the cells which become lysed in the high temperature of the first denaturation step).

The mixture PCR reaction component:

Enzyme buffer (10x)	2 µl
dNTP mix (2mM each of dATP, dCTP, dGTP, dTTP)	1 µl
Forward primer (10 pmol/µl)	0.25 pmol/µl
Reverse primer (10 pmol/µl)	0.25 pmol/µl
Dream *Taq* DNA polymerase	1.25 U
Water nuclease free	up to 20 µl

PCR program:

Initial denaturation	95°C	10 minutes	
Denaturation	95°C	15-30 seconds	25-30 Cycles
Annealing	T_m-5°C	30 seconds	
Elongation	72°C	1 min/kb	
Final Elongation	72°C	2-5 minutes	

The PCR amplification result was visualised via electrophoresis on a 0.8% Agarose gel.

2.4.8 **Sequencing of DNA fragments**

All plasmid constructions were checked for correct and mutation free sequence. DNA sequence analysis was done by Göttingen Genomics Laboratory, (University of Göttingen) using the chain termination method by Sanger (Sanger *et al.*, 1977). Sequencing was also performed according to the protocol recommended by GATC Biotech (Konstanz, Germany). The Sequence Data was analyzed using Chromas Lite, Clone manger 9 and Staden Package programmes.

2.4.9 **Glycerol cultures of the bacteria**

To permanently maintain the bacterial strain harbouring the recombinant vector DNA of interest, culture stock was prepared by re-suspending 750 µl of the sterile 50% glycerol to the same volume of the overnight grown bacterial culture (ratio 1:1 v/v). Glycerol stocks were stored at -80°C.

2.5 Conjugation

A common bacterial mechanism used for genetic exchange in the nature is called conjugation. Studies on the variety of enterobacterial element conjugation have resulted in a model which is considered a two-step process. The component involves bringing recipient and donor cells together to form an effective mating pair. The donor considered for making a pilus called the F+ (fertility) factor, makes a copy of the plasmid and extends the pilus to the recipient F- cells. The next step is the enzymatic transfer of the single strand of the conjugative plasmid which involves the action of a special protein complex relaxosome that produce single stranded cleavage at the specific site (*nic*) within the origin of transfer (*ori*T) of the conjugation element. (Lederberg *et al*., 1946; Lanka and Wilkins 1995). The F-plasmid has *ori*V as the special origin of the replication and *a mob* region which may be transferred by conjugation into *L. lactis* subsp. *cremoris* MG1363 and *Lb. plantarum* WCFS1 strains. The donor such as *E. coli* S17-1 (Simon *et al*., 1983) was used for conjugation which can form the RP4 apparatus.

The following description is based on a cultivation of the bacterial culture in late exponential phase of the growth for both donor-recipient bacteria.

The recipient *L. lactis* subsp. *cremoris* MG1363 strain was inoculated from a single colony into 5 ml MRS culture and was grown overnight at 30°C without shaking. At the same time the donor *E. coli* S17-1 strain harboring the conjugated plasmid DNA carrying the fragment gene of interest was grown in 5 ml LB selective pre-culture with appropriate antibiotics. The following day the main-culture was re-inoculated with the overnight culture and incubated at the optimal growth temperatures for *E. coli* S17-1 and *L. lactis* subsp*. cremoris* MG1363 strain until they attain the late log phase of the growth of about OD_{600} = 0.9 and 1.2 respectively.

Both the cultures were harvested at a defined growth stage at (5,000 rpm for 2 min at RT). The *E. coli* cell pellet from 1 ml culture (OD_{600} of 1.2) was washed twice with LB

medium and the same wash step for the *L. lactis* cell pellets was from 5 ml culture with MRS medium. Both the obtained cell pellets of *E. coli* and *L. lactis* were mixed in 1:3 and 1:5 cell ratio and then resuspended in 0.5 ml pre-warmed medium MRS of the recipient. However, the suspension mixture was pipetted into the middle of the MRS plate which was prewarmed at RT. The plate was incubated at 30°C for 24-28 hours which was the optimal growth condition of the recipient bacteria. Afterwards, the cells of the plate were washed off with 1 ml MRS medium, and different dilution volume of the washed cells were re-plated on MRS-agar medium supplemented with special appropriate antibiotics, one of them that inhibit the growth of donor bacteria and has no effect on the recipient cells. Due to the natural resistance of the *L. lactis* to the polymyin B in contrast to *E. coli*, only *L. lactis* subsp. *cremoris* MG1363 clones bearing the conjugated plasmid can grow on the MRS plate containing polymyin B (40 µg/ml) and additional plasmid specific appropriate selective antibiotic (5 µg/ml Erythromycin).
To disrupt the gene *glgC* in the chromosomal DNA of the target organism *L. lactis* subsp. *cremoris* MG1363, the homologous region of about 700 bp present in the vector construct was used, which had an additional erythromycin cassette beside the Kan system for selection and detection of the single crossover knockout. The successful integration into the chromosome of the non replicated conjugated vector was transferred into *L. lactis* cells and plated on erythromycin MRS selective plates. Only colonies haveing pK18::M-C1-*Em* integrated into the chromosomal DNA are able to grow.

2.6 DNA hybridisation - Southern blot

The southern blot analysis is used to confirm a specific DNA fragment in the genomic DNA of an organism. The completely digested genomic DNA with appropriate restriction enzyme were separated according to their molecular size on 0.8% agarose gel electrophoresis and subsequently transferred to a nylon-membrane (Hybond-N, Amersham).
An amplified PCR product with gene-specific primer or purified fragment of the digested knockout vector that was used for disruption the gene was labelled with Biotin Labelling Kit DecaLabel DNA (Fermentas, Vilnius, Lithuania). The reaction for labelling of the probe was performed as recommended in the manufacture´s manual and the labelled probe was used after denaturation in boiling water bath for 10 min to hybridize with a digested genomic DNA of the bacteria.

2-5 µg of chromosomal DNA was completely digested with a restriction enzyme overnight. The enzyme was selected based on obtaining a visible difference in the labelled fragment between the investigated genotype and the wild type.

The digestion reaction:

Genomic DNA		5µg
10X buffer		1x
Restriction enzymes		5-10 U
Water nuclease free	up to	25µl

The digested DNA was separated on 0.8% agarose-gel by electrophoresis at 60 volts for 2 hours and then the gel was stained in ethidiumbromide bath (for short time less than 5 min) documented by GelDoc (Biozyme Innotech) and washed in H_2O_{bidest} before blotting.

A vacuum blotting device (BioRad) was used to transfer the DNA to a nylon-membrane (1 cm larger than the blotting mask). The membrane was washed with distilled water and then the mask was applied and the gel placed on top of the membrane with an attention to remove all air bubbles. The vacuum during the blotting process was kept constant at 6-7"Hg and the following solutions were successively applied directly on the gel according to the procedure:

- Depurinisation with buffer (250 mM HCl): 1x 10 min
- Quickly washing twice with $_{dd}H_2O$
- Denaturation with buffer (1.5 M NaCl and 0.5 NaOH): 2x 15 min.
- Quickly washing twice with $_{dd}H_2O$
- Neutralisation with buffer (3 M NaCl+ 0.5 M Tris-HCl pH 7.5): 2x 15 min.
- Blotting with buffer 20x SSC pH 7.0 (0.3M Na-citrate+ 3 M NaCl): for 2 hour.

All buffers were autoclaved at 120°C for 20 min, and stored at RT. The DNA was fixed by exposure of the membrane to a UV light (λ= 254 nm) for 1min, and the dried between Whatman papers (Blotting Papiere GB02, Schleicher and Schuell) to proceed to the next step, or was stored for long time in aluminum foil at RT.

Hybridization and detection procedure:

Hybridization buffer:	
SSC (20x)	25% v/v
Blocking stock solution (10%)	10% v/v
N-Lauryl sarcosine (10%)	1% v/v
SDS (10%)	0.2% v/v
$_{dd}H_2O$	up to 50 ml

10 % blocking stock solution:	
Blocking reagent	10 g
Maleic acid buffer	up to 100 ml

Maleic acid buffer pH 7.5:	
Maleic acid	1 M
NaCl	1.5 M

Wash buffer 1:	2x SSC+ 0.1% SDS
Wash buffer 2:	0.1x SSC+0.1% SDS

2-Pre-Hybridization buffer (Fermentas):

The Following pre-Hybridization solution was optimized to use at 42°C and prepared to the final concentration.

6x SSC
5x Denhardt´s solution
0.5% SDS
50% v/v deionized formamide

The hybridization was carried out at 42°C and the procedure was used as described in the protocol of the manufacturer´s instructions (Fermentas)

2.7 Protein Extraction

Two methods have been used to extract the target protein from the cells depending on the volume of the cell samples

2.7.1 **French press**

The biological materials (e.g., proteins, amino acids, enzymes) contained inside the cells to be purified or analyzed must first be released from the cell using a process called 'cell disruption' or 'lysis'. Cell disruption is the first step in the purification and analysis process. Therefore, French press is one of the most critical steps affecting the yield and quality of the results. The internal cylindrical pressure of the French cell press increases the pressure of the biological sample (American Instrument Company, Silver Spring, USA). As the intracellular pressure increases and the cells are dispensed through the sample outlet tube, the external pressure on the cell walls drops rapidly towards atmospheric pressure. The pressure within the cell drops as well but not as quickly as the pressure external to the cell. This pressure differences causes the cell wall membrane to burst thereby releasing the intra-cellular contents. The free cellular components can be collected and separated as required.
As the presence of the thick layer of peptidoglycan in Gram-positive bacteria require additional cell-wall degradation (*Lactococcus* and *Lactobacillius*), the resuspended cells were treated with 10 mg/ml lysozyme to a final concentration of 100 µg/ml and incubated at 37°C for 30-60 min before disruption of the suspended cells.

2.7.2 **Ultrasound**

A different method to disrupt or lyse the cells is invariably required when small culture volumes are used. Thus sample volumes less than 20ml were disrupted with ultra-sonification (ultrasonic processor UP 200S, 24 kHz, 200 W, Dr. Hielscher GmbH, Teltow; sonotrode S_2, 2 mm). The sound waves are delivered using an apparatus with a vibrating probe that is immersed in the liquid cell suspension. Mechanical energy from the probe initiates the formation of microscopic vapor bubbles. When these bubbles reach resonance size, they collapse releasing mechanical energy in the form of shock waves equivalent to several thousand atmospheres of pressure. The shock waves disrupt cells present in suspension. To prevent excessive heating, ultrasonic treatment is applied in multiple short bursts to a sample immersed in an ice bath. This method was applied only to get small amounts of protein extract that allows variable parameters to check and optimize rapidly. The cell suspension was centrifuged in a microcentrifuge tubes; the duration of ultrasound needed depends on the cell type, the sample size and the cell concentration (30-40% amplitude, cycle 0.5). The samples

were cooled on ice during decomposition. The centrifugation of the cell debris was carried out at maximal speed of centrifugation at 13,000 rpm at 4°C for 15-20 min (Biofuge Heraeus, Thermo scientific). The supernatant was transferred to a new clean tube and stored at 4 °C for further experiments.

2.8 Determination of protein concentration

The protein content in the cell lysate was determined in an assay based on the method of Bradford (Bradford 1976). The principle of the Bradford assay is measuring the maximum absorption change of coomassie-brilliant blue reagent (Fermentas) at 595 nm, upon binding to proteins, which can easily be monitored by photometric analysis. The 1 ml of the Bradford reagent was added to 5-10 µl of appropriately diluted cell lysate in 1 ml photometer cuvette (Sarstedt, Nümbrecht, Germany). After 5 minutes incubation in the dark, OD_{595} was determined. Values or volumes of further experiments were adjusted relative to the measured OD_{595} of the different samples. The protein concentration was calculated based on a bovine serum albumin (BSA) standard curve. The protein concentration was calculated by the following equation:
Protein concentration mg/ml = value OD_{595} * Bradford (BSA) factor/volume of the sample (ml)

2.9 Protein Analysis

2.9.1 SDS polyacrylamide Gel electrophoresis (SDS-PAGE):

The preparation of protein fractions were analysed by SDS-PAGE (Laemmli, 1970). The analyses are based on the separation of proteins according to their molecular weight during traversing through the gel pores under the effect of electric-current.The proteins were separated in vertical gel electrophoresis apparatus (Bio-Rad) filled with electrophoresis buffer. The separating and stacking gel compositions are described below. Polymerization was started by adding ammonium persulfate (APS 10%) and catalyst TEMED, shortly vortexed and poured in between the glass plates according to the manufacturer´s instructions. Finally, the gel was overlaid with dd$H_2O$and it takes about 20-30 minutes to complete the polymerization. The water was removed and stacking gel was poured on the top of the separating gel. The comb was placed to form the 10 wells for applying the protein samples. The electrophoresis unit was assembled

as recommended by the manufacturer and the chamber was filled with 1x electrophoresis buffer.

Before loading into the wells, protein samples were mixed with SDS loading buffer and heated for 5-10 min at 95°C to denature the proteins and complex with SDS molecules. Then the heated samples were cooled on ice and centrifuged at high speed for 30 secs. The supernatant was applied with about 10-15 µg of protein amount on the gel. The electrophoresis was performed at constant current 15 mA per gel for 20 min and then switched to 30 mA and continued for about 1 hour until the blue dye reached at the end of the gel. Afterwards, the gel was either stained with coomassie blue or silver stains or subjected to western blot analysis. Protein Molecular Weight Marker was separated on the same gel used to estimate the molecular weight of analyzed protein, (Fermentas, Vilnius, Lithuania).

30 %(w/v) Acrylamide–Bis (SERVA)

2 %(w/v) bromophenol blue: in ddH_2O, stored at 20°C

Tetramethylethylenediamin (TEMED) (Plusone, Amersham Bioscience)

(TEMED is used with APS to catalyze the polymerization of acrylamide)

10% APS (w/v) was dissolved in water and aliquoted (500 µl) and stored at -20 °C.

Separation buffer:

Tris	18.2g
SDS	0.4g
$_{dd}H_2O$	up to 100 ml

Adjusted the pH to 8.8 with 1 M HCl

Stacking buffer:

Tris	6.1g
SDS	0.4
$_{dd}H_2O$	up to 100 ml

Adjusted the pH to 6.8 with 1 M HCl

10X Electrophoresis buffer:

Tris	30.3g
glycine	144.1g
SDS	10g
$_{dd}H_2O$	up to 1000 ml

4X SDS loading buffer (sample buffer):

Glycerol	7.5 ml
Tris	0.4g
SDS	1.2g
Bromophenol blue 2% (w/v)	0.5 ml
β-Mercaptoethanol**	2.5 ml
$_{dd}H_2O$ up to	50 ml

** It is not essential in case of prokaryotic protein

SDS-PAGE gel preparation:

Table .9: The Composition of the SDS gel

	Separation gel		**Stacking gel**
	10%	12%	4%
Separating buffer	1 ml	1 ml	-
Stacking buffer	-	-	0.48 ml
AA (40%)	1 ml	1.2 ml	0.2 ml
$_{dd}H_2O$	2 ml	1.8 ml	1.32 ml
APS (10%)	30 µl	30 µl	15 µl
TEMED	3 µl	3 µl	2 µl

This volume is calculated for one gel. The separation protein depends on the pore size which is determined by the amount of the acrylamide in the gel. High acrylamide concentrations form narrow pores that are required for the separation of low molecular weight proteins and vice versa.

2.9.2 **Coomassie Blue Staining**

Coomassie Blue staining method (Meyer and Lamberts, 1965) was used in order to detect the separated protein bands in the gels. Gel was submerged in coomassie-blue staining solution for 30-60 min to overnight at room temperature that allowed the dye to bind the proteins. Afterwards, gel was submerged in the destaining solution to remove the excess of the dye. The gel was incubated in batch of the destaining solution with gentle shaking at RT until the background became transparent. The gels were scanned and documented using TMA1000XL, ScanMaker 1000XL (MICROTEX).

Coomassie Blue staining solution:	
Coomassie blue R (SERVA)	0.05%
Acetic acid (v/v)	10%
Isopropanol	25%
Destaining solution:	10% acetic acid

2.9.3 **Silver staining (Blum *et al*., 1987).**

Silver staining was used to visualize and detect a low amount of the protein bands in SDS-PAGE. After electrophoresis the gels, as described above, were incubated with different solutions. The gel was incubated with the fixation solution at RT for 2 h to overnight with gentle agitation. Subsequently, the gel was washed three times with washing solution (30% ethanol) for 20 min and then washed twice quickly with deionized water, followed by incubation with 0.02% sodium thiosulphate for 1 min, and then for 20 min in 0.2% silver nitrate solution. Further, the sample was re-washed twice with $_{dd}H_2O$ and incubated in developing solution for less than 5-10 min until the protein bands were visualized. Afterwards, the gel was immediately washed twice for 1 min each time with $_{dd}H_2O$ and incubated for 5 min with stop solution. Finally, the gel was placed for 30 min in $_{dd}H_2O$.

Fixing solution: 40% ethanol, 10% acetic acid

Developing solution: 3% sodium carbonate, 0.05% formaldehyde (37%)

Stop solution: 0.5% Glycine

The other commercial procedure used for silver staining followed the recommended manufacture instructions of the kit (Fermentas).

2.9.4 **Western Blot (Towbin *et al*., 1979. Modified):**

After the separation of the proteins by SDS-PAGE, proteins were transferred from the gel onto Hybond-nitrocellulose membranes (Amersham Biosciences) in the presence of the blotting buffer. For that, the membrane was activated by washing in water and equilibrating for several minutes in blotting buffer. The gel was placed on the membrane and covered on both sides by three layers of the Whatman filter paper soaked in blotting buffer. Blotting conditions were calculated for each 1 cm^2 of the gel to give 5 milliampere (mA) and electrophoresis for 60-90 min. The membrane was washed in water (deionization) and blocked for 60 min at room temperature or overnight at 4°C in TBS buffer containing 3% BSA (w/v). In order to detect the proteins, the membrane was incubated at RT for one hour with TBS buffer containing anti His-tag (rabbit) (1:5000) and then washed three times with water for 10 min each time. Washing can be done with TBS as well. Subsequently the membrane was incubated in TBS buffer containing the secondary high affinity antibody IgE for 30 min with shaking. The membrane was again washed three times with TBS buffer for one hour and incubated in DIG detection buffer. The protein band was visualized by incubating the membrane with BCIP solution (100 µl NBT and same volume of BCIP in 10 ml DIG buffer). The detection reaction was stopped by washing the membrane with water.

TBS buffer:

Tris-HCl	2.42g
NaCl	8.7 g
$_{dd}H_2O$	up to 1000 ml

Adjusted the pH to 7.5

Blocking Reagent: TBS buffer with 3% (w/v) BSA

DIG detection buffer:

NaCl	5.84g
$MgCl_2$	1.02g
Tris	12.11g
$_{dd}H_2O$	up to 1000 ml

pH adjusted to 9, storage the buffer at 4°C.

Transfer buffer for Blotting:

Tris	6g
Glycine	29g
Methanol	400ml
$_{dd}H_2O$	up to 1000 ml

The buffer was stored at 4°C.

2.10 Methods for protein expression and purification

2.10.1 Heterologous protein expression

A- Escherichia coli

Large-quantity expression of recombinant protein is usually essential for the functional and structural protein analysis of the target gene. A variety of gene expression systems have been developed. The pET101 expression system was chosen for high production of the gene of the interest and for its ability to enable the quick production of large quantity of protein in *E. coli* BL21 (DE3). The system would be activated when Lactose sugar or lactose analogue isopropyl-β-D-thiogalactopyroside (IPTG) was added to the media which binds to the repressor and induces its dissociation from the operator permitting transcription from the promoter. The cloning was performed as recommended in manufacturer by Invitrogen.

The transformation mixture was transformed into Top10 chemical competent cells. Positive plasmid from Ampicillin-resistant colonies were identified with restriction analysis (plasmid purified from 4 ml overnight LB culture using mini prep QIAgen plasmid purification Kit) and correct constructs were confirmed by sequencing the DNA to check for mutation and proper integration of the PCR insert.
The correct plasmid construction of the gene was transformed to a special expression *E. coli* strain BL21 (DE3). A single colony was picked and incubated in LB medium containing appropriate antibiotic (100 µg/ml ampicillin) overnight at 37°C with shaking (at least 150 rpm). A fresh 30 ml culture was inoculated with the overnight culture. When the cells were in the mid-log phase with an OD_{600} about 0.6-0.7 they were induced for different time points 1, 2, 3, 4 h and overnight with IPTG to a final concentration of 0.1 mM at 37°C with shaking 150-200 rpm. The samples of un/induced

and control were harvested by centrifugation at maximal speed for 5-10 min and re-suspended with 20-50 mM Tris-HCl buffer pH 8. Subsequently, the cells were disrupted via ultrasonic or French press, respectively according to the volume of the samples which were 30 ml or 1000 ml. The supernatant fractions were obtained from the debris cells by cold-centrifugation at high speed for at least 15-20 minutes at 4°C. To facilitate the separation and visualization, overexpression of the recombinant protein process was analysed with polyacrylamide gel electrophoresis (SDS-PAGE) with, the crude extract, supernatant and pellets fractions having 10-20 µg proteins applied on the 12% gel. As a control the crude extract of the strain was used for the respective construct without induction or other construct carrying small PCR fragment or foreign protein (as Cellulose-ORF). The gels were stained with coomassie blue to visualize the increasing intensity of proteins bands corresponding to the expected size range.

A1- Co-expression of the gene with chaperones

Chaperones are proteins that assist the non-covalent folding or unfolding and assembly and disassembly of other macromolecular structure. Expression of foreign proteins in *E. coli* often results in various problems, like the formation of the inclusion bodies. These unfolded proteins will aggregate in insoluble protein granules form in the cytoplasm. Molecular chaperone have been confirmed and supported to help in the process of protein folding. The co-expression experiment was performed by the manufacture supplied instruction manual.

The *E. coli* Rosetta 2 strain (Ros 2) used for coexpression contains the chaperone plasmids C1-5 set from Takara. The construct pET101-WCFS1-*glgCD*$_{his}$ was electro-transformed into *E. coli* Ros 2 C1-5 and the culture at the middle of log-growth phase at OD_{600} about 0.6-0.8 for 4 h was induced for different chaperones under the recommended concentration of 0.4% L-arabinose and 10 ng/ml tetracycline as shown in the Chaperone Takara kit (Tab.10).

Table.10. Takara chaperone set used in current study.

Plasmid	Chaperone	Promoter	Inducer	Resistantce marker
C1: pG-KJE8	dnaK-dnaJ-grpE groES-groEL	*ara*B *Pzt*1	L-Arabionse Tetracycline	Cm
C2: pGro7	groES-groEL	*ara*B	L-Arabionse	Cm

C3: pKJE7	Dnak-dnaJ-grpE	*ara*B	L-Arabionse	Cm
C4: pG- Tf2	groES-groEL-tig	*Pzt*1	Tetracycline	Cm
C5: pTf16	Tig	*araB*	L-Arabionse	Cm

A2- Expression and Purification of protein pMAL Fusion Maltose Binding Protein (MBP)

The gene of interest was cloned in pMALc$_2$x protein fusion plasmid downstream from *malE* gene with specific primers that were designed to amplify the gene without start codon and with a modified cutting site to facilitate the cloning and cleavage with a specific protease F.Xa. This gene encoding maltose binding protein (MBP) has been proven to enhance the solubility of protein expressed in *E. coli*. All cloning, expression and purification steps were performed as recommended by the manufacturer´s instructions manual (BioLabs, New England). The only modification in this step was the amylose resin which was prepared in the laboratory by washing the amylose sugar with autoclaved milliQ water for at least 10 times. 2-5 ml of amylose resin was packed in the special column (MoBiTec, Göttingen 2.5 and 10 ml volume) and 20 mg resin in the FPLC column. The flow rate was controlled to be not more than 1ml min^{-1} and 0.4-0.5 ml min^{-1} in MoBiTec and FPLC column, respectively. The column was equilibrated and washed with 1x bed volume of the column buffer for 1x and 4x times, respectively. The fusion of the maltose binding protein (MBP) with the target protein was eluted with elution buffer. The elution fraction was collected and dialyzed against target buffer (1xLEW for His-Tag purification or 20 mM Tris-HCl) at 4°C overnight or by using the concentration fraction tube (Sartorius, Göttingen, Germany) with buffer exchange. The protein composition was cleaved with Factor Xa protease (New England Biolabs) with the optimal enzyme buffer (20 mM Tris-HCl pH 8.0 with 100 mM NaCl and 2 mM $CaCl_2$) according to the manufacturer´s instructions. All the purified fractions were collected and analyzed on 12% SDS-PAGE gel

Column buffer:

20 mM Tris HCl pH 7.4
200 mM NaCl
1 mM EDTA

Elution buffer:

20 mM Tris HCl pH 7.4
200 mM NaCl
1 mM EDTA
100 mM Maltose

B *Corynebacterium glutamicum*

New methods were developed to introduce new vectors into bacteria *C. glutamicum*, allowing the gene expression and the analysis of promoters and terminators (Liebl *et al*., 1989a; Schläfer *et al*., 1990; Liebl *et al*., 1992). The high G+C, Gram-positive *C. glutamicum* with expression plasmid pWLQ2 was used in this work to express the interesting genes *glgC* and *glgD* from *Lactococcus lactis* and *Lb. plantarum* WCFS1.

C *Bacillus subtilis* 168

Production of foreign proteins in *B. subtilis* has been technically difficult, in part because of the inherent structural instability of most Gram-positive plasmids carrying recombinant DNA insert. The shuttle pHCMC02, 04 and 05 carrying weakly constitutive promoter P_{lepA}, xylose inducible promoter P_{xylA} and IPTG-inducible promoter P_{spac}, respectively (BGSC: Bacillus Genetic Stock Center) were used to express the genes *glgC* and *glgD* from *L. lactis* subsp. *cremoris* MG1363 and *Lb. plantarum* WCFS1 (For more information Nguyen *et al*., 2005).
The DNA genes were amplified using primer with PCR program for each gene and the purified PCR product from 0.8% agarose gel were inserted into multiple cloning sites of the plasmids. The pHCMC02 did not succeed and the purification from harbouring *E. coli* strain had a lot of problem, as well as the cloning into pHCMC04 did not succeed for unknown reason. Successfully, the genes *glgC* and *glgD* were cloned into pHCM05 and transformed into *E. coli* XL1. The purified construct was analysed by restrictioni enzymes and DNA sequencing and the correct plasmid construct, free of mutation was naturally transformed into *B. subtilis* 165. The construct was re-purified and checked with different restrictions enzymes before overexpression step.

2.10.2 **Homologous gene expression.**

Lactic acid bacteria (LAB) are widely used in the food industrial fermentation. Number of genetic tools, including transformation protocols, cloning vectors (Ravn *et al*., 2000; Poquet *et al*., 1989). Mutagenesis system and protein expression have been developed for LAB (Dieye *et al*., 2001). High level production of proteins in LAB has been obtained using *L. lactis* constitutive promoters (Kuiper *et al*., 1997; de Vos, 1999).

To circumvent the problem of high level expression in *E. coli* and some other bacteria which usually lead to aggregation or degradation of the protein in cytoplasm or periplasm homologous expression systems have been developed. As it consists only of genetic elements of the relevant bacterium, it will be advantageous for the facilitation and range of use. Inducible expression promoters have been developed where the gene expression can be controlled by an inducer like most commonly used controllable expression system, nisin-controlled gene expression (NICE).

2.10.2.1 Mechanism of the Nisin Controlled gene Expression system:

The 34 amino acids peptide antimicrobial Nisin, is used for induction of the two-component signal transduction (regulatory cascade).

The nisin cluster is controlled by the two-component regulation system including a sensor kinase (NisK) and response regulator (NisR) (Kuipers *et al*., 1995).

At the start the nisin molecule bind to the membrane-bound histidine protein kinase which resides in the cytoplasmic membrane (encoded by *nisK*). Upon binding NisK gets autophosphorylated at a conserved His residue and transfer the phosphate group to the intracellular response regulator NisR which leads to activate the regulator. This in turn induces the nisin operon at the nisin A promoter (*Pnis*A) controlling the gene of interest (de Ruyter *et al*., 1996; Kuipers *et al*., 1995). The NICE system cleverly exploits this autoregulatory mechanism by cloning the *nis*-RK regulatory genes, along with the promoter nisA onto separate plasmids. In these fusion constructs, host LAB strains containing the NICE plasmid system can be induced to express and over-produce homologous as well as heterologous proteins by adding of sub-inhibitory amounts of nisin (0.1–5 ng/ml) to the culture medium (de Ruyter *et al*. 1996). Depending on the presence or absence of the corresponding targeting signals, the protein is expressed into the cytoplasm or secreted into the medium (Fig .13).

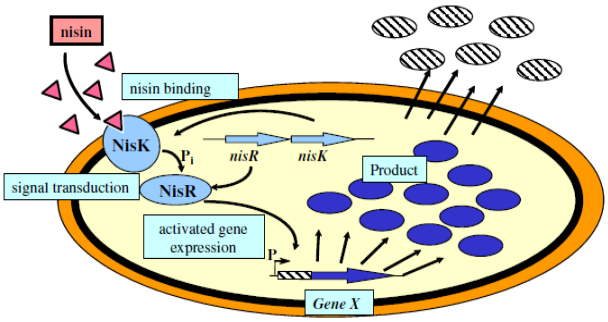


Fig.13 Nisin-controlled gene expression. *NisK* indicates histidine–protein kinase; *NisR*, response regulator; and Gene X, the target gene to be cloned behind the nisA promoter with or without targeting signals (de Ruyter et al. 1996).

2.10.3 **Concentration of Protein by ultra-filtration**

The purified protein samples were concentrated using Vivaspin ultrafiltration system up to 15 ml (Satorius, Göttingen, Germany) at maximal centrifugation speed of 3,000 rpm.

2.10.4 **Purification of Protein with Fast Protein Liquid Chromatography (FPLC)**

All buffers and samples were filtrated. Samples were filtrated using 0.2 µm or 0.45 µm filter (Sartorius), buffers were produced using milliQ water and filtrated using vacuum pump with cellulose nitrate membrane filter (0.2-5 µm ,Φ 47 Whatman, Germany). The column was washed with filtrated H_2O_{bidest} and stored at 4°C in 20% (v/v) ethanol

Affinity chromatography

The target protein GlgC fused into MBP in the expression vector pMAL-W-*CD*_$_{his}$ was purified based on the affinity of the MBP to amylose sugar and high specific affinity to maltose. The column was packed with amylose resin, equilibrated and washed with MBP column buffer and the complex fusion protein eluted with maltose elution buffer at the flow rate 0.3- 0.5 ml/ min.

MBP column buffer:	20 mM Tris-HCl pH 7.4
	200 mM NaCl
	1 mM EDTA
Elution buffer:	MBP column buffer + 100 mM Maltose

2.11 Methods for Enzyme Assay

2.11.1 **Determination of Enzyme Activity**

A different assay was used to detect the ADP-glucose pyrophosphorylase enzyme activity

1-High-performance liquid Chromatography (HPLC) assay:

The HPLC was carried out using CarboPac PA-1 column (Dionex, Sunnyvale, CA). The following solvents were used as elutes: 1 mM sodium hydroxide (E1) and 1 M sodium acetate in 1 mM sodium hydroxide (E2). An aliquot of the enzyme reaction (10-30 µl) was injected in the column equilibrated with mixture (80:20, v/v) of E1 and E2. Elution was performed by the following gradient:
T_0 = 1 ml/min (75%E1+25%E1); T_5 = 1 ml/min (45%E1+55%E1); T_{20} = 1 ml/min (45 %E1+55%E1); T_{30} = 1 ml/min (20%E1+80%E1); T_{35} = 1 ml/min (100%E2). Nucleotides and sugar nucleotides were detected by absorption at 260 nm.

2-Chemical activity assay:

A- Indirect enzyme assay: The activity of the enzyme ADPPase was measured depending on the determination of the glucose-1-phosphate consumption in synthesis of the ADP-glucose reaction. The formation of NADH could be measured at 340 nm UV light as described by Takata *et al*., 1997. The reaction mixture (600 ml) contained: 100 mM Tris (pH 7.5), 15 mM $MgCl_2$, 15-50 mM ATP, and 5-10 mM glucose-1-P and enzyme solution. Two blank controls were used; blank1 did not contain enzyme and blank 2 did not contain ATP and/or glucose-1-P. The reaction was incubated at 25-30°C and was further stopped by heating the mixture at 100°C for 2 min and then cooled on ice. Subsequently, 300 µl of the reagent mixture was added to the reaction mixture.
The reagent mixture contained: 100 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$ and 1.8 nM NAD^+, 6 U per ml of phospho-glucomutase PGM (from rabbit muscle; Boehringer Mannheim, Mannheim, Germany), and 6 U per ml of glucose-6-phosphate dehydrogenase G6PDH (from *Leuconostoc* sp. Boehringer).
The mixture was incubated at 37°C for 30 min, and the NADH concentration was measured at A_{340} (Fig.14)

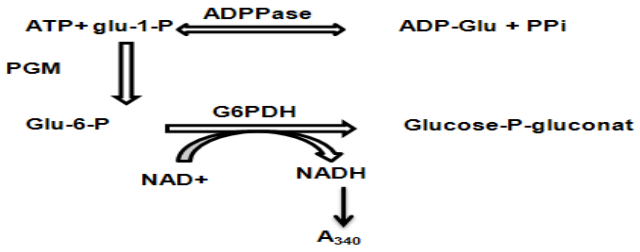


Fig.14 schematic show the activity assay of the ADPPase based on measuring the consumption of the glu-1-P and the formation of NADH is measured at A340 nm.

B- Colorimetric method for the assay of ADP-glucose pyrophosphorylase (C.Fusari *et* al., 2006).

In this method the released inorganic orthophosphate, Pi, was quantified and measured after specific hydrolysis of PPi by inorganic pyrphosphatase. Pi dosage is performed by the technique based on formation of a phosphomolybdate- malachite green complex (Hess and Derr, 1975; Lanzetta *et al.,* 1979). The activity of the enzyme ADPPase is measured in the direction of the synthesis of the ADP-glucose and PPi reaction. The reaction mixture (900 ml) contained: 100 mM Tris (pH 7.5), 15 mM $MgCl_2$, 15-50 mM ATP, and 5-10 mM glucose-1-P and 10% of the final concentration of the supernatant/enzyme solution coupled with 1 U of inorganic pyrophosphatase enzyme as one step reaction. The reaction was incubated at 25-30°C and was further stopped by adding the complex reagent MG-am (ammonium molybdate + malachite green complex) or it was stopped by heating the mixture at 100°C for 2 min and then added the inorganic enzyme incubated at 25°C as recommended. Two blank controls were used; blank1 did not contain enzyme and blank 2 did not contain ATP and /or glucose-1-P, (Fig.15)

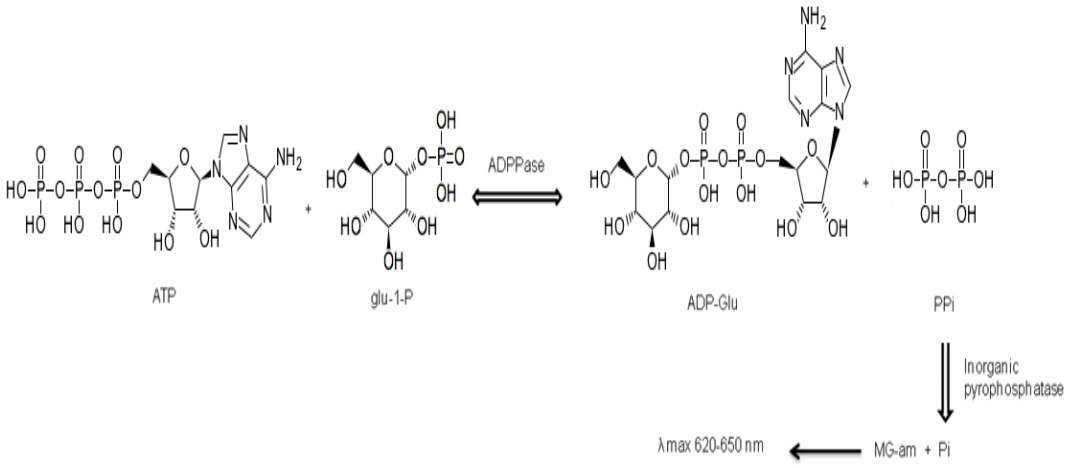


Fig.15 schematic the activity assay of the ADPPase using MG-am reagent.

2.11.2 **Thin Layer Chromatography (TLC)**

TLC was used to separate different compounds based on their mobility difference in two-phases namely the mobile (hydrophobic) and the stationary (hydrophilic) silica gel. 10 µl of the sample were applied to 1 cm lines on silica gel aluminum (20x10cm) plate (TLC Silica gel 60 F_{254}, MERCK, Germany). The plate was developed in a sealed chamber (lined with Whatman paper and the cover greased with Vaseline), saturated with100 ml of the solvent solution until the solvent forehead reached 70–80% of the

plate's height. Afterwards, the plate was dried and the developing step was repeated for better separation. Then the TLC plate was dried and spots were visualized with freshly prepared developing solution and exposure to heat at 110-120°C for 15 min in an aluminum foil.

Solvent solution	
1-propanol	50%
Nitromethan	30%
$_{dd}H_2O$	20%

Developing reagent	
Aniline	1 ml
Diphenylamine	1 g
Acetone	100 ml
Stored in dark at	4 °C

Developing solution consisted of developing reagent plus 0.1 vol. of 85% Ortho-phosphoric acid (5ml+0.5 ml enough for one plate).

2.11.3 **3-amino, 5- Dinitrosalicylic acid assay (DNSA)**

This method measures the presence of free carbonyl group or reducing sugars released during the enzymatic breakdown of polysaccharide.

The reaction:

3, 5-Dinitrosalicylic acid (750µl) +Enzyme reaction (Yellow) $\xrightarrow[\text{Phenol, } Na_2SO_3]{\text{100°C, 15 min}}$ 3-Amino-5-nitrosalicyl acidic (brown)

In the process, the DNS reagent turns to brown colour in the presence of reducing sugar which absorbs light strongly at 575 nm.

DNSA-Reagent

3, 5-Dinitrosalicylic acid	10.0 g
Phenol	2.0 g
Na_2SO_3	0.5 g
K-Na-Tartrate	200.0 g
NaOH	10 g
$_{dd}H_2O$	add to 1000 ml

2.12 RNA techniques

2.12.1 **Extraction of RNA**

RNA isolation and purification was performed according to the manufactures instructions of MasterPure complete DNA & RNA Purification Kit (EPICENTER Biotechnologies). The RNA was eluted with free RNase and sterile (2x autoclaved) H_2O_{bidest}. The purified RNA was stored at -70°C. (All prepared solution must be RNase free).

2.12.2 **Measurement of the RNA concentration**

The RNA concentration and the purification of RNA were determined using NanoDrop spectrophotometer ND 1000 (Peqlab Biotechnology).
The quality of extracted RNA was analyzed by electrophoresis 5 µl of the RNA extract on 0.8% agarose gel together with the DNA marker followed by ethidium bromide staining. Any smearing of bands indicated to the degraded mRNA, when new sample of total RNA was prepared.

2.12.3 **PCR-Reverse Transcription RNA (RT_PCR**)

The first strand cDNA was synthesed by a specific Reverse Transcriptase enzyme from the purified RNA and then was amplified by PCR. The reaction had been carried out according to the described manuals recommended by RevertAid H minus First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania).

To characterise and analyse their functions, *glgD* and *glgC* were separately and simultaneously expressed in various host microorganisms including Gram-negative *E. coli* and Gram-positive *B. subtilis* and *C. glutamicum* for heterologous expression and in *L. lactis* subsp. *cremoris* MG1363 under control of the NICE expression system for homologous expression. Also, attempts to overexpress the genes in question in glycogen free lactic acid bacteria strains like *Lactobacillus delbrueckii* subsp *bulgaricus* and *Lactobacillus sanfranciscensis* were also made.

3.1 Heterologous expression of lactic acid bacterial genes *glgC* and *glgD* and other LAB genes

3.1.1 *Heterologous expression in Escherichia coli BL21*(DE3):

3.1.2 Cloning and expression of the glgC and glgD the genes with the pET101 system

Over production of the proteins GlgC and GlgD of *lactococcus* and *Lactobacillus* species were carried out at first as heterologous-expression in *E. coli* BL21 based on the high expression system pET which has a strong T7 promoter. In this step, the genes were amplified using KOD DNA polymerase wherein the primers were designed with a specific overhang for easy cloning and correct orientation determination. The PCR reaction was carried out as described before in material and methods section at the annealing T°55-60°C. The amplified product was confirmed on an 0.8% analytical agarose gel and the DNA fragment was purified and cloned into the pET101 plasmid as described.

The strategy was focused on expressing each gene of each strain to characterize the protein function separately and simultaneously to understand and detect the correlation or the interacting function between proteins especially because the target *glgC* and *glgD* genes are overlapped (Fig. 17).

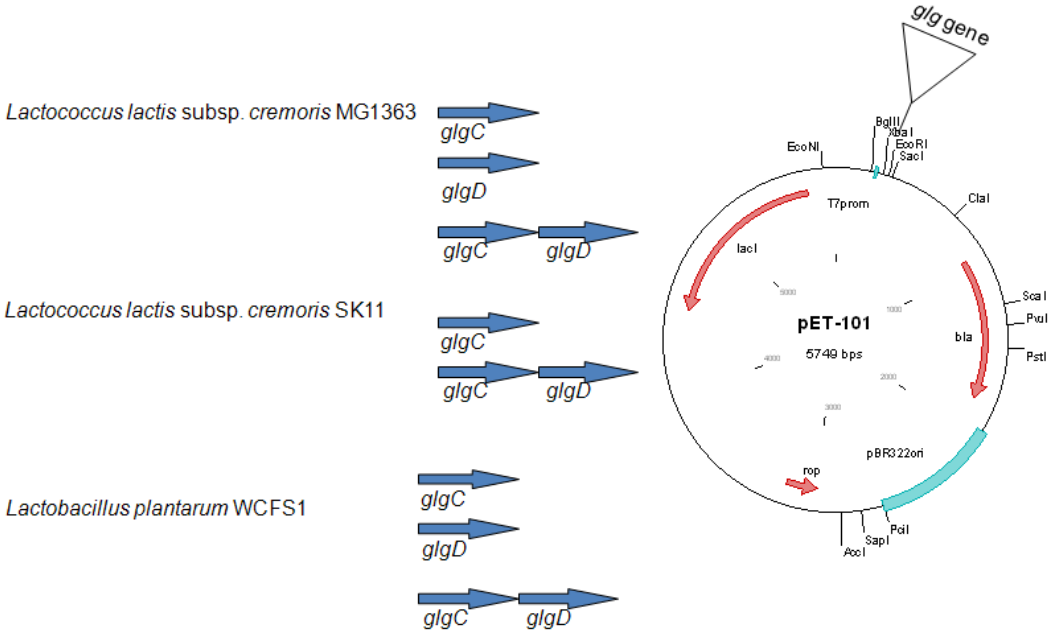


Fig .17. The cloning and expression strategy for genes of interest from different lactic acid bacteria species by using pET101 TOPO expression system in *Escherichia coli.*

The overexpressions of the proteins in *E.coli* BL21 were detected as insoluble aggregates in the cytoplasm and/or periplasmic space of the cells as inactive proteins. A Western blot analysis was performed to confirm the previous detection of the overexpression bands in the insoluble pellet fraction by SDS-PAGE. No anti-polyhistidine–tag signal was detected in the supernatant fraction which indicates that a non-soluble protein form was expressed even at low concentration (Fig.18).

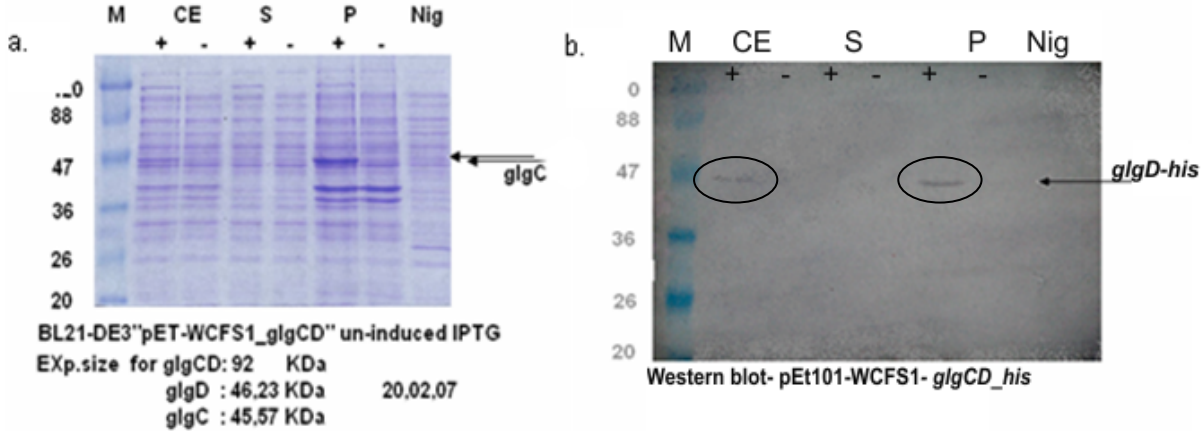


Fig. 18 **A**; **heterologous expression of *glgC* and *glg D***. SDS-PAGE gel of 4 h expression genes *glgC* and *glgD* from *Lb. plantarum* WCFS1 in *E. coli* BL21 (DE3) carrying the plasmid pET101-WCFS1-*glgCD*$_{his}$. Marker (M), crude extract (CE) induced with IPTG (+), un-induced CE (-), induced Supernatant (S+), un-induced Supernatant (S-), induced pellet (P+), un-induced pellet (P-), control. **B**; Western blot analysis of the genes *glgC* and *glgD* expression against anti-histidine. The signal was detected in the induced pellet which refers to expression the genes inactive form as inclusion bodies.

S-soluble extract; CE-crude extract; P-pellet extract and Nig- negative control with empty vector pET101.

[+] and [-] IPTG

To reduce the frequency of inclusion body formation and increase the soluble fraction of the target protein expression was carried out at low-temperature 30°C and 25°C. In addition to growth temperature, the coexpression of molecular chaperone was used which can increase the percentage of soluble protein and facilitate the purification. Growing cells under stress in the presence of ethanol help in some situation to reduce the inclusion bodies and increase the solubility.

3.1.3 **Co-expression the *glg* genes with chaperones genes**

This approach involving the coexpression of the tagged protein with different kinds of chaperones, or cofactors was used to improve the refolding or/and solubility of the protein product. Several chaperones were tested to find the optimal partner for the protein of interest. Different vectors coding for various of chaperones were used for the chaperone plasmid kit from Takara.

However, the coexpression with the vector DNA pET101-WCFS1-g*lgCD*_his with different chaperone C1-5 under standard growth condition did not provide any soluble protein fraction as shown in the Fig 19.

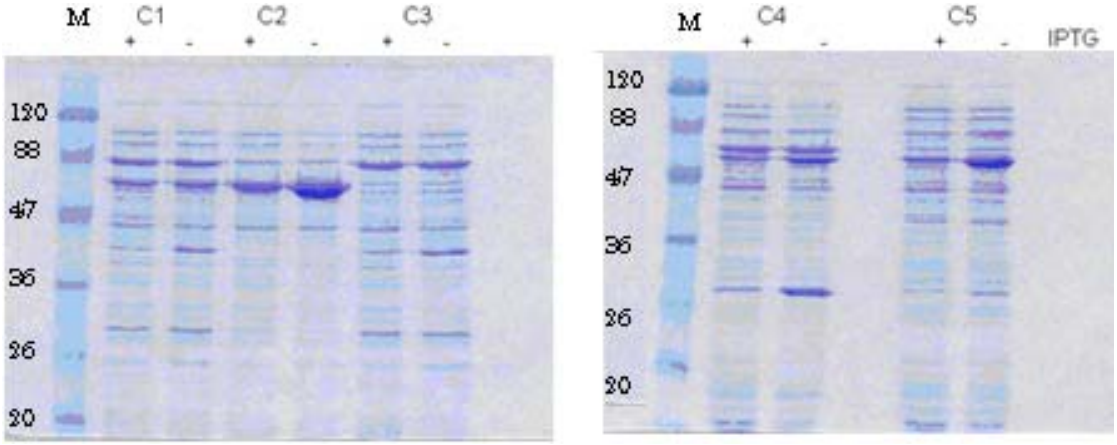


Fig. 19. Visualization the SDS-PAGE gels with coomassie blue stain of coexpression interesting genes *glgC* and *D* in pET101-WCFS1-*glgCD* construct with different chaperones (C1-5) from Takara. [+] and [-] induced/un-induced as a control

3.1.4 **Expression of the genes under stress conditions**

3.1.4.1 Low temperature

As mentioned before, a widespread strategy to reduce the aggregation of recombinant protein consists of cultivation at low temperature. Thus, in some cases it has been proven effective in increasing the solubility of the protein and reducing the formation ratio of the inclusion bodies. (Sorenson *et al*., 2005)(Huke S. Betton, J. M., 2003).

E. coli BL21 carrying the expression vector pET101 with different target-genes was cultured at low temperature 30 and 25°C after induction with different amounts of IPTG at final concentrations of 0.1 and 0.05 mM with strong shaking at more than 160 rpm for 4 h as shown in Fig 20. There was no decrease in the aggregation of the inclusion bodies at this temperature or increase in the soluble recombinant protein. Furthermore, no soluble protein in the supernatant fraction was detected by SDS-PAGE after further polyhistidine-mediated affinity purification under native conditions in case of hints for slight soluble expression (data not shown).

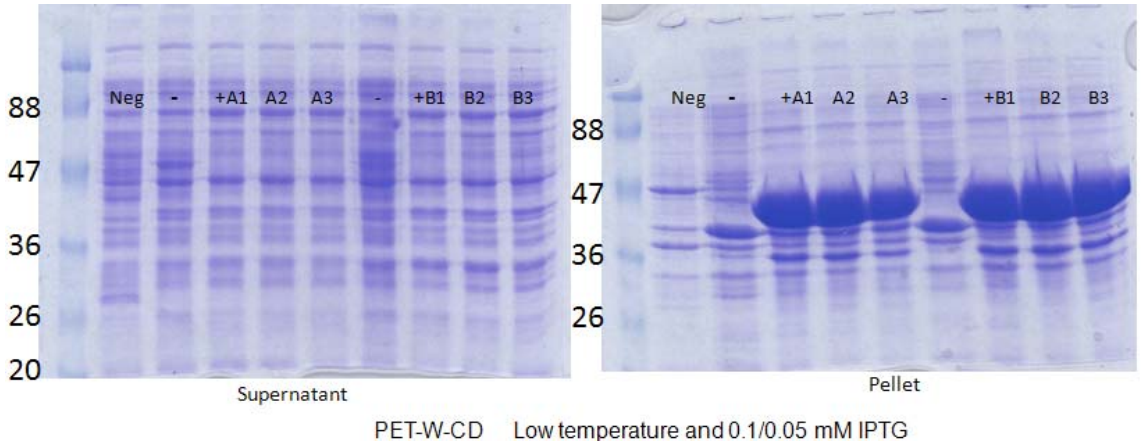


Fig. 20 SDS-PAGE gel showing the expression in *E. coli* Bl21 (pET-WCFS1-*glgCD*) at different low temperature point and different IPTG concentration +1A; induced 0.1mM at 30°C for 2h. +2A ; 0.05 mM IPTG at 30°C for 2 h. +3A; 0.1 mM IPTG at 25°C for 2h. +1B; 0.1 mM IPTG at 30°C for 4h. +2B; 0.05 mM IPTG at 30°C for 4 h. +3B; 0.1 mM IPTG at 25°C for 4h.

3.1.4.2 Expression under stress condition

Growth and induction of the *E. coli* BL21cells under osmotic stress in the presence of salt, sucrose and ethanol has been shown to increase the level of soluble fraction of recombinant protein (Bowden *et al*., 1988; Blakwell *et al*., 1991).

The cells of *E. coli* BL21 harbouring the expression vectors pET101-WCFS1-*glgC* and/or *glgD* have been cultured overnight with selective antibiotic at final concentration of 100 µg/ml ampicillin and was used to inoculate fresh LB medium. After 30 min of incubation at 37°C, 99% of ethanol was added to the medium prior to inoculation at a final concentration of 3% (v/v), followed by an incubation at 37°C until the cells reached the middle of log-phase OD_{600}= 0.6-0.8, when they were induced with IPTG to a final concentration of 0.1 mM. The cells were harvested after 4 h of induction. Further processes were carried out as described previously. Under this condition, no increase in the protein solubility was absorved as shown in Fig. 21.

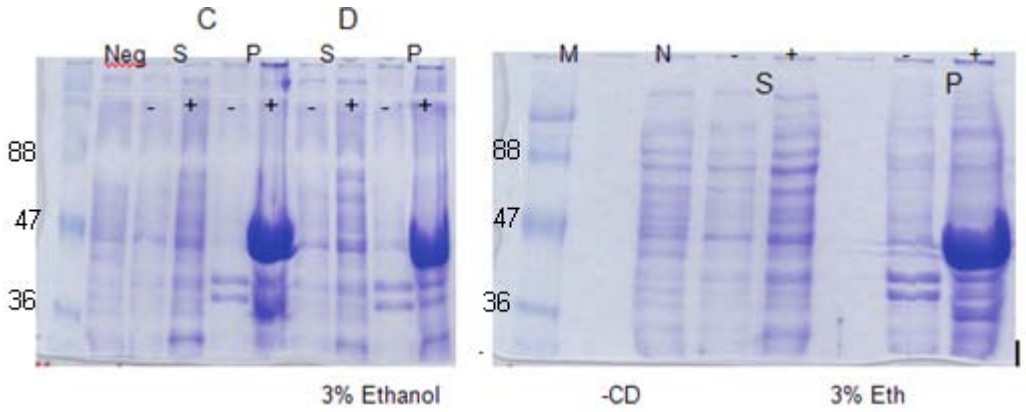


Fig. 21 SDS-PAGE gel showing the expression in *E. coli* BL21(pET101-WCFS1-*glgC*/*D*/*CD*) under stress condition, 3% of the EtOH was added to the cells before induces with IPTG to final concentration 0.1 mM and for 4 h incubated at 37 °C. Neg/N: negative control. Supernatant (S) [+] and [-] IPTG Pellet (P) [+] and [-] IPTG

3.1.5 **Refolding and solubilised of inclusion bodies**

Different procedures were used to purify the inclusion bodies proteins in soluble and refolded form using different buffers, but the refolding efficiency was either always too low or zero resulting in incorrect refolded protein.

As mentioned before, the expression of the target genes in *E. coli* BL21 under T7 control lead to the accumulation of incorrectly folded protein as inclusion bodies in the pellet. The cells were expressed under the native condition as described above and disrupted with sonication. After centrifugation, the polyhistidine-tagged protein, which was expressed as inclusion bodies, is extracted from the pellet fraction and solubilised using the denaturant (8 M urea or 6 M Guanidine-HCl). Further, the extract was clarified by centrifugation and applied on the Ni-column. The washing and elution of polyhistidine-tagged protein under denaturing condition was performed according to the instructions provided by the manufacturer. The elution fraction was used for further refolding protein experiment. The purification step was analyzed on 12% SDS-PAGE gel (Fig. 22)

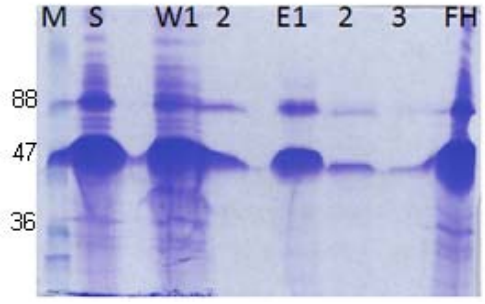


Fig. 22 Histidine purification under denature condition. M: Marker, S: supernatant of the pET-WCFS1-*glgC* expression under native condition, W1-2: washing the soluble inclusion bodies on the Ni-column, E1, 2 and 3 first three elution fraction of the polyhistidine-tagged protein, FH: through flow the soluble supernatant fractions

The protein refolding process had been carried out using the ProFoldin 96-Well Protein Folding Plate (ProFoldin, Westborough MA). The elution fraction of the soluble inclusion bodies was applied on the plate and refolding reaction was performed as described in the manual of the manufacturer´s instructions. The enzyme activity assay mixture was added at the end of the refolding procedure to analyse the correct refolding condition carried out using standard assay of ADP-Glc-PPase (Material and methods, section 2. 11. 1) with one modification in the reagent mixture containing the substrate 1.5 mM Iodonitrotetrazolium chloride (INT) as colour detector which allow to directly measure the change of absorption at 492 nm due to reduction of INT. (Fig. 23)
The plate was scanned with spectrophotometer and showed colour change in different spots A1,-2, and -6; C/E/G -1 and -6 with high intensity at level A1, -2, C1 and E6. Sample of these spots were analyzed with HPLC but did not show any activity or any difference in the signal compared with the negative spot as a control. An explanation of these changes in the colour may be that despite some reaction between the INT detector substrate with buffer contents in the plate spots, there was no correct refolding of the soluble inclusion bodies.

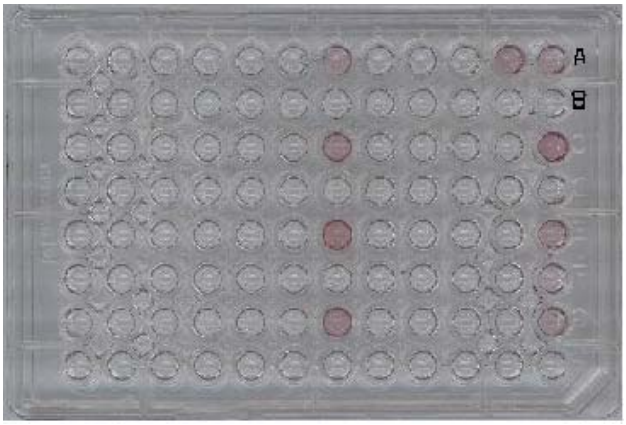


Fig. 23. Showed the activity test after using ProFoldin 96-Well Protein Folding Plate.

3.1.6 **Expression in different *E. coli* strains and other LAB genes**

The Gram-negative bacterium *E. coli* is the most commonly used organism for heterologous gene expression. Disadvantages of high level over-expression are that the recombinant proteins are accumulated in huge quantity either in cytoplasm or periplasmic space in the inactive form as inclusion bodies. Rare codon or frequency may especially cause problems for high level expression. A host with similar codon usage is optimal for expression in *E. coli* like BL21 plysS, codon plus and Rosetta strains were attempted to reduce this problem.
The expression vector pET101-*glg* genes (pET101-MG-*glgC*/*D*/*CD*, SK11-*glgC*/*CD* and

WCFS1-*glgC*/*D*/*CD*) was also expressed in different *E. coli* strains Rosetta 2, BL21 (DE3) plysS and Arctic Express no soluble fraction of the proteins were detected in this step also (Fig. 24).

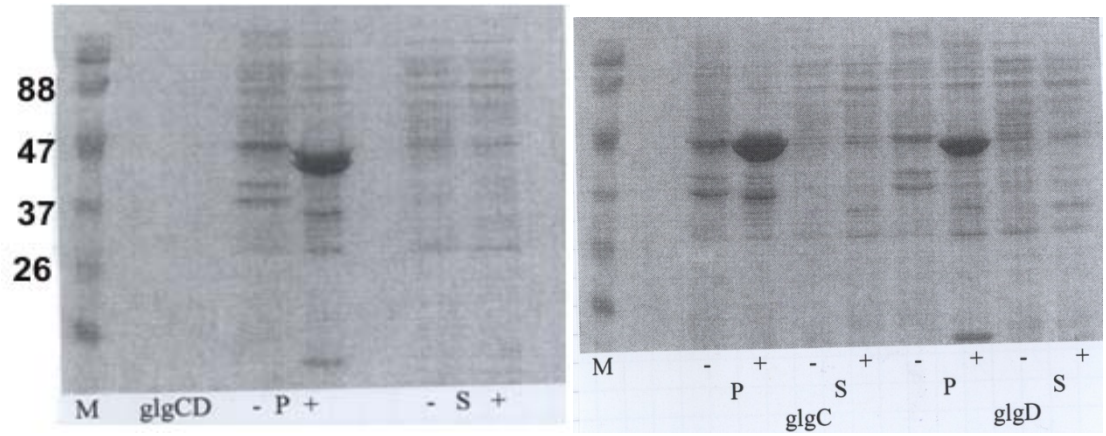


Fig. 24. The SDS-PAGE gel showing the expression in *E. coli* Rosseta strain carrying the plasmid pET-WCFS1-*glgC*/*D*/*CD*_his. Protein Marker (M) Pellet (P) [+] and [-] IPTG Supernatant (S) [+] and [-] IPTG

3.1.6.1 Heterologous expression of other Lactic acid bacteria genes

The interesting ORF1: 174434-172767-ncontig from *Lb. sanfraciscensis* 1.1304 is a 555 amino acid sequence (~ 64.2 KDa). It was classified as an alpha-amylase of the glycosyl hydrolases family 13 from amino acid homology analysis (Pfam).

These ORF for characterization of the biochemical function has been cloned in pET101 expression vector expressed in different *E. coli* strain BL21 (DE3), BL21 (DE3) plysS which carries the lambda DE3 lysogen. In addition, BL21 (DE3) pLysS cells contain the pLysS plasmid, which constitutively expresses T7 lysozyme. T7 lysozyme reduces the basal expression of target genes by inhibiting T7 RNA polymerase. This provides tight control of T7 RNA polymerase which is necessary for the recombinant protein to be expressed as toxic, The strain *E. coli* Arctic Express have been engineered to improve protein production at low temperature and has a cold chaperone *Cpn*10 and *Cpn*60 from *Oleispira Antarctica* which have a high similarity about 74% to 54% amino acid to the GroEL and GroES, respectively. The proteins show a high refolding activity at temperature of 4-12°C and increase the recombinant protein solubility.

The supernatant and pellet fractions of ORFs expressed in *E. coli* BL21 plysS under different condition factors (IPTG, Glucose concentration and antibiotic) was analyzed on 12% SDS-PAGE electrophoresis gels resulting in the aggregation of the protein as inclusion bodies (Fig. 25a). It was noticed that the expression of the target protein of the ORF1 or other in *E. coli* BL21 (DE3) accumulated higher amount of the protein

(inclusion bodies) compared to the expression amount which has been obtained from *E. coli* BL21 (DE3) pylsS strain (Fig. 25b)

A

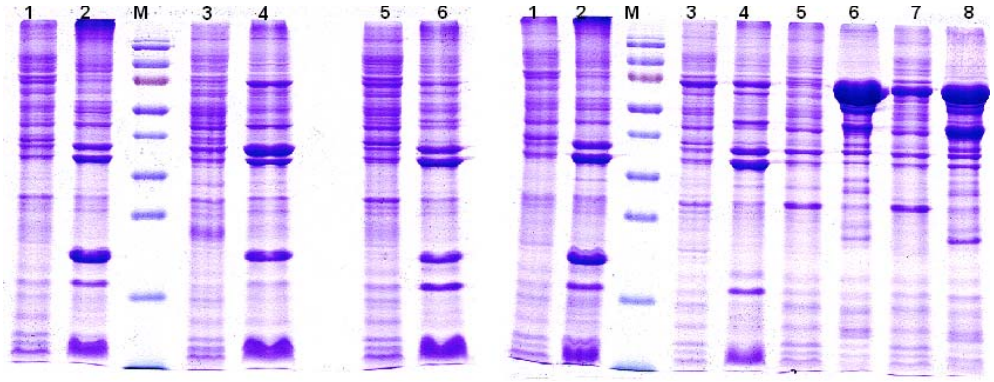


Fig. 25 A. SDS-PAGE gel showing the expression in *E. coli* BL21-pLysS carrying the expression plasmid pET101-san-α-Amylase. Lane: 1and 2 control un-induced Supernatant (S) and Pellet(P); lane 3 and 4 induced with 0.05 mM IPTG S and P, respectively; lane 5 and 6 induced with 0.1mM IPTG plus 1% glucose. B: lane 1and 2: control un-induced. Lane 3 and 4: 0.025 mM IPTG+1% glucose+ Amp (Ampicilin).lane 5 and 6 induced 0.05 mM IPTG. Lane 7 and 8: 0.1 mM IPTG+1%glucose+Amp S and P, respectively.

B

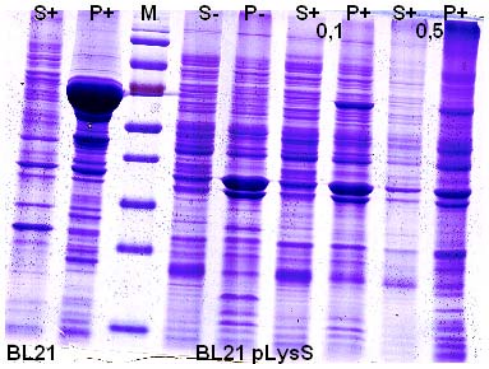


Fig. 25. B; expression in *E. coli* BL21 (DE3) and plysS. Protein Marker (M), Pellet (P) [+] and [-] IPTG, Supernatant (S) [+] and [-] IPTG (0.1 and 0.5 mM)

The target ORF1 was cloned and expressed under heterologous condition by the Nisin controlled system using the plasmid pMSMP3535 in *L. lactis* MG1363.

The primers F.amy._xbaI and R.amy_Bam (50 bp dowm stream of the stop codon) were used to amplify the ORF1 from the purified genomic DNA of *Lb. sanfraciscensis* 1.1304 and 1.54. The PCR product was obtained just from the subspecies 1.1304 and there was no product from 1.54, under optimal PCR parameters with annealing temperature 65°C. The amplified product was cloned into plasmid pMSMP3535 opened with the same *Bam*HI and *Xba*I that were used for preparative digestion of the purified PCR product from 0.8% agarose gel. The resulting plasmid vector construct p35-San-*Amy* was transformed into *E. coli* XL-10 and analyzed with restriction enzymes. The correct construct was transformed into *L. lactis* MG1363 by electroporation and

selected on MRS-Agar plate supplemented with erythromycin to a final concentration of 5µg/ml. The vector pMSMP3535 was transformed also to use as a negative expression control.

The ORF1 was expressed in *L. lactis* MG1363 harbouring the vector p35-San-Amy under the control of NICE system, 5 ml of MRS or GM17 broth was inoculated with overnight culture under selective condition, and induced with Nisin to final concentrations of 0.1, 1, 3 and 10 ng/ml. When the culture reached the beginning of the log- growth phase at optical density 600 nm between 0.3-0.4, 2 ml of the induced culture was collected at different time points after 1, 2, 3, 4 h and overnight by centrifugation at 5.000 rpm for 5 min at 4°C. The cell pellets were resuspended in 20 mM Tris-HCl pH7.4 and incubated with lyzosyme for 30-60 min at 37°C before disrupting the cells by ultra-sonication (30 amplitude, 0.5 Cycle and at 4°C for 5-10 min). The crude extract and supernatant fractions were analyzed on 12% SDS-PAGE gels and the intensity of the protein bands were developed with coomassie staining (Fig. 26).

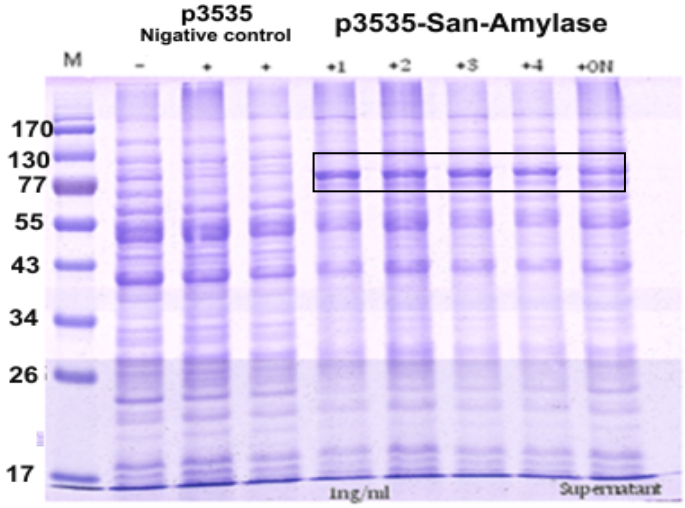


Fig. 26. Supernatant fractions induced with nisin to final concentration 1 ng/ml and induced for different times point1-4 hr and O.N, of heterologous-expression *L. lactis* MG1363 harbouring the nisin plasmid pMSMP3535 carrying the ORF1 from *Lb. sanfranciscensis* strain. The black box show the increasing in the intensity of the expression the construct carrying the ORF 1(Amy) under different nisin concentration comparing with the control carrying the empty plasmid pMSP3535. The control (empty vector) was induced for 4 h and overnight

The supernatant fraction of the ORF1 expressed under NICE system was used for further TLC experiments to analyse the affinity of ORF1 to substrate. Different substrates (starch, trehalose, dextrin10, α/ γ cyclodextrin, maltose, amylopectin, amylose, pullulan and glycogen) were incubated with the supernatant fraction at 25-30 °C room temperature for 30 and 60 minutes and 10µl of the reactions were loaded on TLC Silica gel Aluminum (10x20cm) plate (TLC Silica gel 60 F_{254}, MERCK, Darmstadt,

Germany) (section 2. 11. 2, TLC section in material and methods). No affinity of the ORF was detected (Fig.27). Moreover, no significant reducing sugars have been determined or free glucose when measured in reaction performed according to the DNSA-assay and the manufacture´s manual of kit D-GLUCOSE-HK (Megazyme, Ireland), respectively.

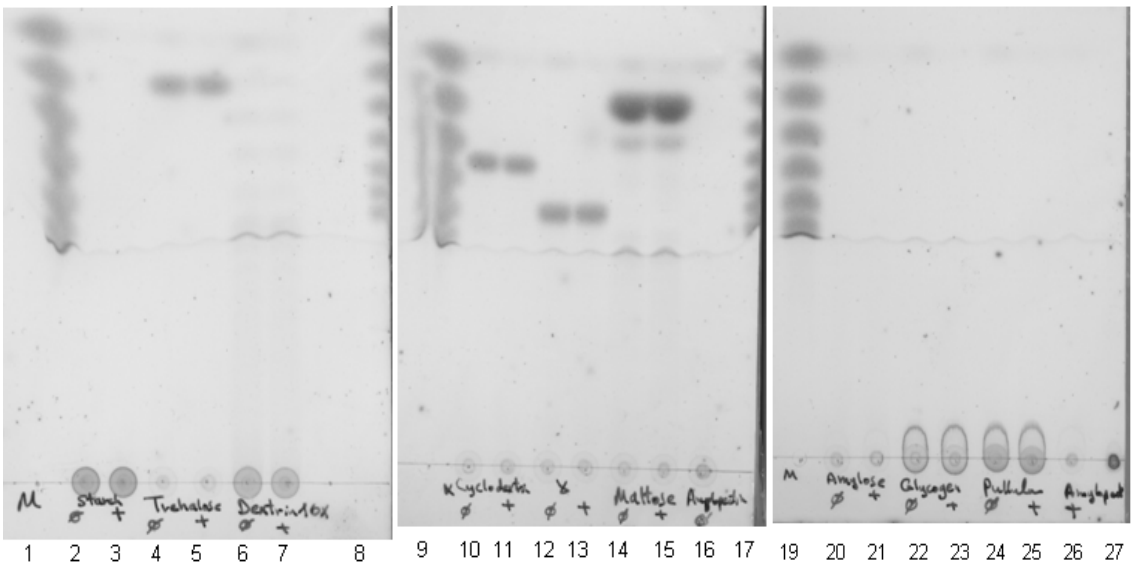


Fig. 27 TLC analyse of the ORF 1 from *Lb. sanfranciscensis* with different substrate to screen the activity of the ORF1. The supernatant fractions were incubated with different substrate for 30 minutes at room temperature then and load 5 µl of the reaction on the TLC Platte.

Lane 1, 8, 9, 17 and 19 maltooligosaccharide standard (M) C1-7

Lane 2 and 3 starch [+] and [-] enzyme

Lane 4 and 5 Trehalose [+] and [-] enzyme

Lane 6 and 7 Dextrin 10 [+] and [-] enzyme

Lane 10 and 11α cyclodextrin [+] and [-] enzyme

Lane 12 and 13 γ cyclodextrin [+] and [-] enzyme

Lane 14 and 15 Maltose [+] and [-] enzyme

Lane 16 and 17 Amylopectin [+] and [-] enzyme

Lane 20 and 21 Amylose [+] and [-] enzyme

Lane 22 and 23 Glycogen [+] and [-] enzyme

Lane 24 and 25 Pullulan [+] and [-] enzyme

Lane 26 and 27 Amylopectin [+] and [-] enzyme

3.1.7 **Expression and purification of (Maltose Binding Protein) MBP-Fusion expression system pMAlc$_2$x**

3.1.7.1 Cloning and expression under standard condition

The genes *glgC* and *glgD* from *Lb. plantarum* WCFS1 have been amplified together by pfu DNA polymerase using special designed primers mal.F and mal.R introducing enzyme cut site *Xmn*I, *Bam*HI, respectively. The vector pET101-WCFS1-*glgCD*$_{\_his}$ was used as a DNA template. The obtained blunt end PCR product was digested with restriction enzymes *Xmn*I and *Bam*HI and ligated in vector pMALc2x that cut with the same enzyme to get the pMALc2x-W-*glgC* and *glgD*$_{\_his,}$ as shown in the Fig.27a.

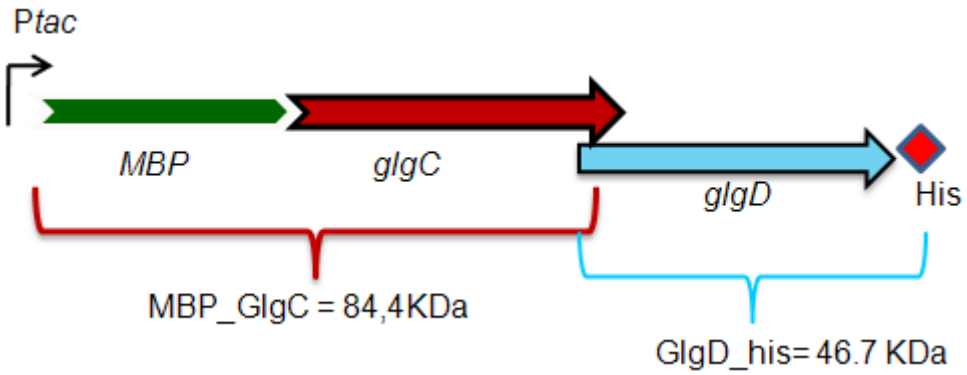


Fig.27a. Schematic shwoing the cloning and expression strategy used co-expression of molecular chaperone increase the percentage of soluble protein and facilitated the purification of the fusion target protein GlgC with the high soluble protein MBP and complex expression with *glgD*$_{his}$ from *Lb. plantarum* WCFS1 in *E. coli* BL21.

The ligation mixture was transformed into cloning strain *E. coli* XL1 blue and the construct was confirmed with restriction analyse and DNA sequencing. The vector was then introduced into *E. coli* BL21 (DE3) and was expressed under the control of the *Ptac* promoter which has been induced with 0.1 mM IPTG after the cell culture reached the middle of exponential phase OD_{600}= 0.5-0.7. Empty plasmid pMAL2cx was transformed into *E. coli* BL21 strain and expressed under the same condition where it was used as a negative expression control for further experiment. The predicted molecular mass of the fusion tagged protein GlgC(MBP-GlgC protein) was about 84.4 KDa and GlgD$_{his}$ protein was in range of 46.7 KDa in case of simultaneous expression.

However, the resulting over-production of fusion MBP-tagged GlgC and GlgD$_{his}$ *Lb. plantarum* WCFS1 proteins in *E. coli* BL21 (DE3) under standard expression condition at 37°C, 150 rpm were analysed by SDS PAGE, the presence of the fusion complexed protein of the expected molecular mass was obtained as an insoluble protein (Fig. 28).

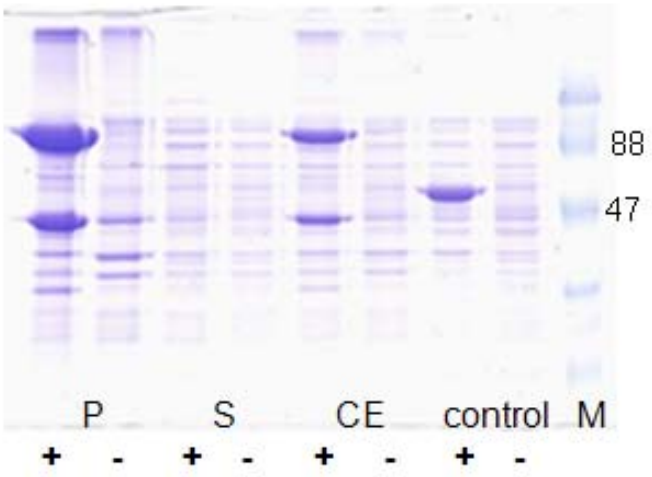


Fig. 28 SDS-PAGe gel showing the overexpression of the Fusion-target protein in *E. coli* BL21 (DE3) harbouring vector pMAL2cx-WCFS1-*glgCD*$_{his}$ under the standard expression condition at 37°C for 4hours, the Fusion MBP-tagged protein GlgC and protein GlgD_his was not detected in soluble fraction, but accumulated in the periplasmic space (induced pellet) as an inclusion bodies form. P: pellet, S: supernatant, CE: crude extract and control is empty plasmid pMAL2cx. (+); induced cells samples with IPTG to final concentration 0.1mM. (-): un-induced samples.

3.1.7.2 Expression and purification under specific condition

Different stress treatments including ethanol and with or without antibiotic, glucose and/ or growth condition at low temperatures 30-19°C have been accepted for the expression of the MBP-tagged complex *glgC* and *glgD*$_{his}$ to give rise to an increase of the soluble proteins form. Indeed, soluble protein fractions were obtained using methods of different mixed stress factors and conditions at the same time. In this method, the *E. coli* BL21 (DE3) culture harbouring fusion expression pMAl2cx-WCFS1-*glgCD*$_{his}$ was inoculated with overnight ampicillin selective LB culture and incubated for one generation about 20-30 min at 30°C without adding any selective antibiotic. Thus, for this reason, the idea should work under super clean condition. The culture was exposed to stress condition by adding 99% ethanol to a final concentration of 2-3 % and growth was continued at 37°C until OD_{600}= 0.6-0.7. The cells were then induced with 0.1M IPTG to a final concentration of not more than 0.05 mM and further incubated with strong aerobic condition of at least 150 rpm at lower temperature 30 °C for maximal of 4 h. The expression fractions were analysed by SDS-PAGE. This mixed treatment gave substantial yield of soluble protein in more than 50%. It did not just increase the soluble Fusion protein MBP-GlgC (Fig.29) but also another distinct band was detected after purification step which corresponded to the molecular size of the GlgD$_{his}$ in soluble form.

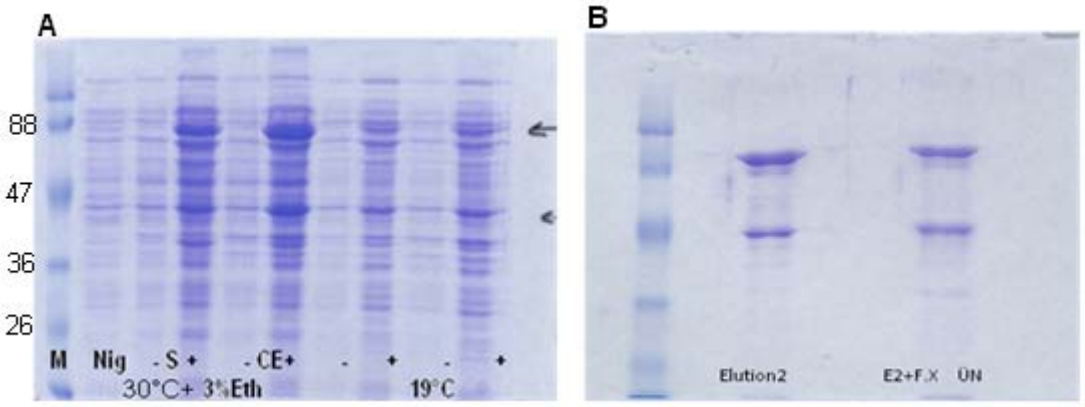


Fig. 29 A. SDS-PAGE gel showing the overexpression of Fusion MBP-target complex protein *glgC* and *glgD*_his from *Lb. plantarum* WCFS1 in *E. coli* BL21 (DE3) under mix of different stress condition and low temperature without any selective antibiotic for not more than 4 h. About 40% of the produced protein was obtained in soluble fraction in the supernatants under mix stress condition and when just low temperature 19°C was used. B; show the soluble fraction of protein overproduction after purification on affinity –Amylose chromatography.

Protein Marker (M) CE-crude extract [+] and [-] IPTG. Supernatant (S) [+] and [-] IPTG .

Mass Spectrometric (MS) and Western Blot analyses confirmed that the band is over produced in soluble form of GlgD_his as shown in Fig 30.

The presence of the GlgD_his in the eluted fraction of the affinity amlyose purification step indicates some affinity and/or interaction between proteins GlgC and GlgD. This interaction was established using poly-Histidine purification to confirm that the two proteins interact together and not that the GlgD protein has some kind of affinity to the amylose matrix. The supernatant was loaded on polyhistidine-column chelating Ni-IDA (Protino, Macherey.Nagel). Washing and elution steps were performed according to the protocol provided by the manufacturer. The molecular mass of the proteins were in agreement with the expected ones as determined from the SDS-PAGE electrophoresis gels analysis (Fig. 31).

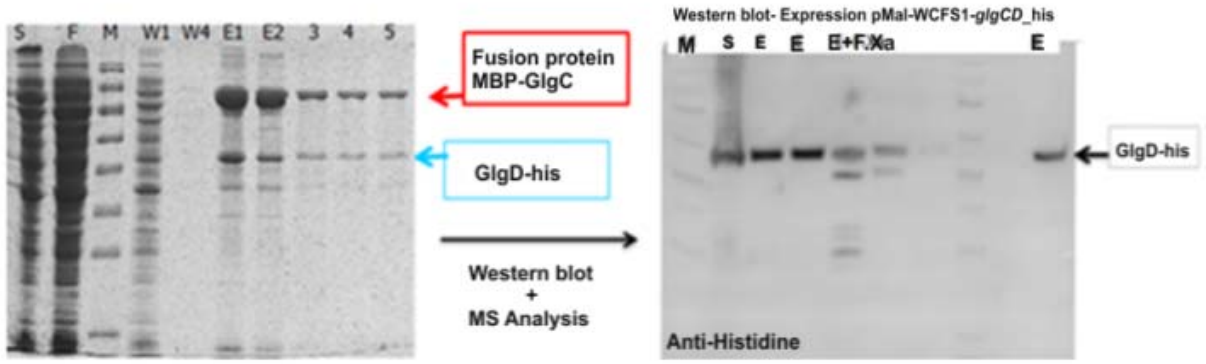


Fig. 30 SDS-PAGE gel stained with coomassie blue of the fusion expression and FPLC purification fractions of fusion MBP-GlgC in *E. coli* BL21-pMAL-WCFS1-*glgCD*_his with affinity chromatography using Amylose column, the expected size range for fusion-target protein corresponding with upper band, the second lower band was expected to be *glgD*_his protein. B: Western blot analysis protein fractions before and after Factor Xa cleavage to detect and confirm the expecting product protein GlgD_his by detecting the signal with antibody anti-His from Roche.S-soluble fraction(

supernatant); CE-crude cellular extract; F- flow through fraction; W-wash fraction; E-elution fractions and E-F.Xa elution fraction after Factor Xa cleavage

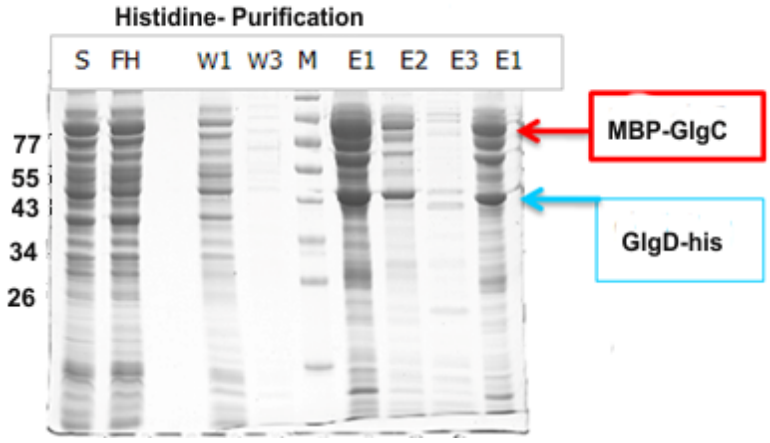


Fig. 31 SDS-PAGe gel showing the Histidine purification used to confirm the corresponding interaction of the two proteins GlgC and GlgD_his.

The supernatant samples were further purified with a FPLC amylase affinity chromatography using property of MBP to bind maltose for elution of the fusion complex MBP-GlgC. Matrix amylase was used to purify the complex proteins in soluble fractions and finally eluted with maltose with the flow rate 0.5 ml min^{-1} (Material and methods section Fusion expression 2.10.1.2A).

In order to separate the target protein, a specific protease Factor Xa (NEB) is used to cleave the fusion product. The cleavage reaction was done after dialysis of the elution buffer of the purified protein fraction with special membrane tube (Visking dialysis tubing 20/32 diameter: 16mm, SERVA) against target buffer 20 mM Tris-HCl pH 7.4 at 4 °C overnight or by using the concentration fraction tube (Sartorius, Göttingen, Germany) with buffer exchange. The reaction was carried out using optimal F.Xa portease buffer (20mM Tris-HCl pH8.0 with 100 mM NaCl and 2 mM $CaCl_2$) at 4 °C for 2-3 days. The molecular mass of the proteins obtained after cleavage was in agreement with the expected one determined from the SDS-PAGE analysis. The gel was stained with coomassie blue for visualization of the increasing intensity band in the expected size range for the recombinant proteins in soluble form. (Fig.32)

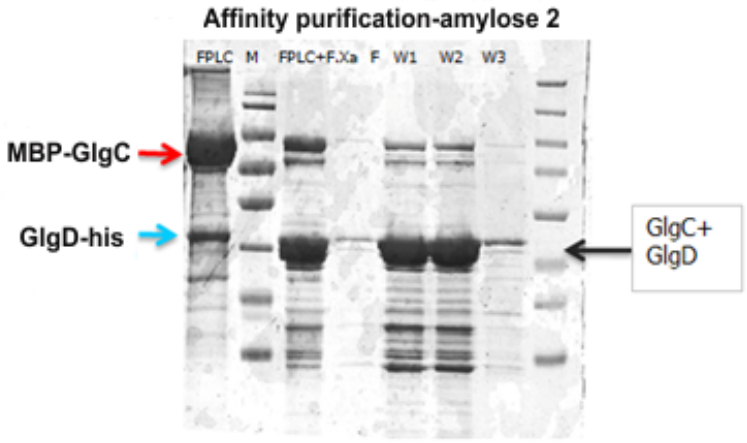


Fig. 32 SDS-PAGe gel showing the second affinity amylose purification for the purified complex fusion MBP-GlgC and GlgD_his after Factor Xa cleavage at 4 °C for 48 h.

The previously obtained results from the affinity amylase chromatography, polyhistidine affinity purification, Western blotting and MS analysis have shown the presence of both the overproduced proteins MBP-GlgC and GlgD_$_{his}$ in the SDS-PAGE. These results strongly confirm our assumption that the two proteins have a high affinity/ interaction to each other to form the ADP-Glc-PPase active enzyme and suggest that the enzyme has an α/β heterotetramer type structure (GlgCD complex). The schematic diagram of the interaction between the proteins GlgC and GlgD is shown in Fig.33

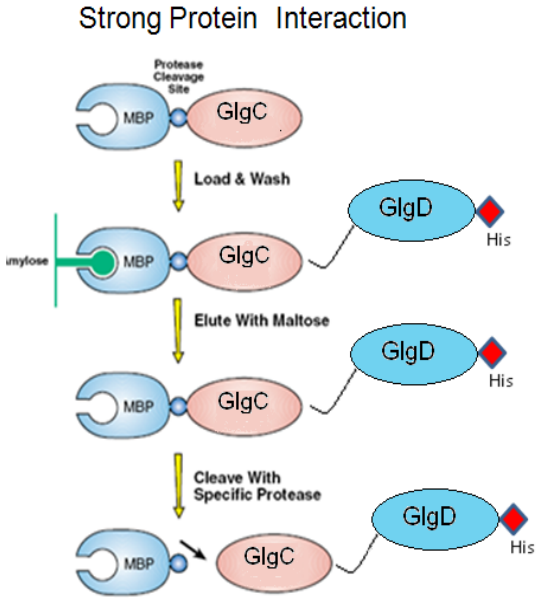


Fig. 33 Schematic showing the expected hypothetical interaction and the high affinity between the two proteins GlgC and GlgD in *Lb.plantarum* WCFS1

3.1.7.3 Establishment the protein: protein interaction

To establish the experimental evidence of the interaction between both of the GlgC: GlgD proteins. New strategies were worked on the first one with the difference in the purification of the two interacting proteins under natural and denature condition. The

complex fusion target protein MBP-GlgC and GlgD_his was purified on polyhistidine-tagged resin from supernatant fraction of the complex stress overexpression *E. coli* BL21 as described above. The part of the supernatant fraction was denatured by exposure to high temperature at 95°C for about 3 min and the denatured proteins were collected by centrifugation at 4°C with a maximal speed of 13,000 rpm for 15 min. The pellet was resuspended with 1xLEW buffer supplemented with Urea to a final concentration of 8 M. The Histidine purification under denatured condition was performed according to the protocol provided by the manufacturer (Protino Ni-IDA kit) and analyzed for SDS-PAGE (Fig. 34). It was expected that there would a disappearance of the fusion protein MBP-GlgC protein band from Histidine-purification after denaturation and that the denatured GlgD_his just would bind to the histidine resin column, Under the denatured condition, the SDS-PAGE analysis showed that the intensity of the band of the fusion protein MBP-GlgC ratio to GlgD band has decreased under denatured purification condition compared to the native condition but there is a one-to-one ratio between them. This might refer to the high affinity interaction between the two purified fusion MBP-GlgC and GlgD_his proteins

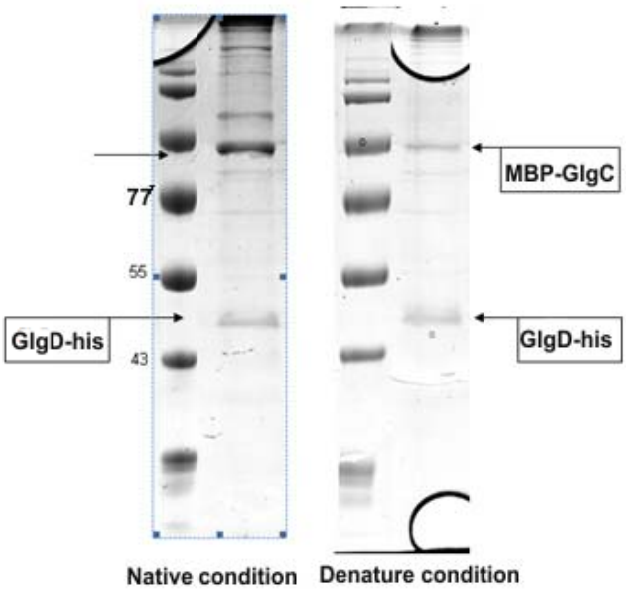
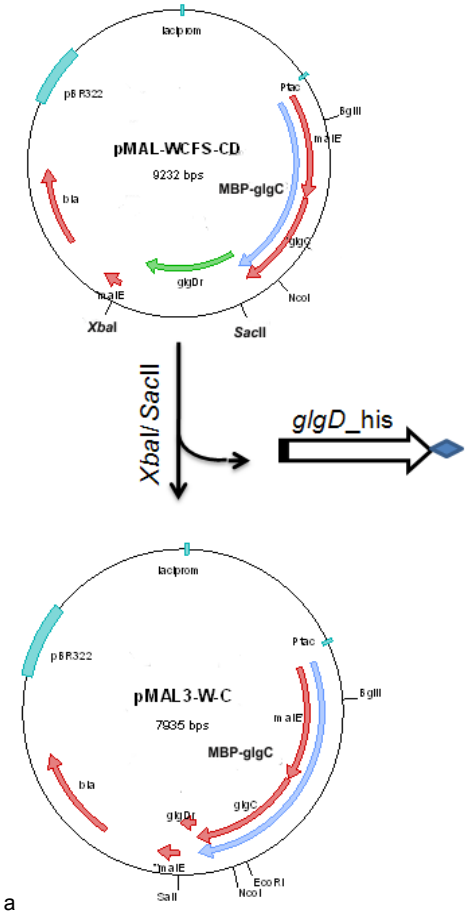


Fig. 34 SDS-PAGE gel showing the Histidine-purification of the complex interaction fusion MBP-GlgC and GlgD_his under native and denature condition.

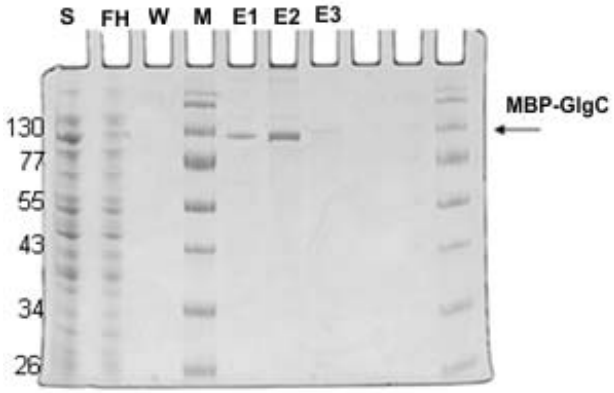
The second experiment was performed to attempt the expression of the target gene *glgC* without *glgD*. This work was done by deleting a fragment of gene *glgD*_his from the expression vector pMAL2cx-WCFS1-*glgCD*_his.

For this purpose, restriction enzymes *Sac*II and *Xba*I give the possibility to cut 1088bp of the (1269bp) gene *glgD*_his, Preparative double-digestion reaction was carried out for

4 h at 37°C and the linearized vector was purified on 0.8 % preparative agarose gel. The new purified construct was religated after modifying the 5´-3´ DNA ends with T4 DNA polymerase. The ligation reaction with T4 DNA ligase has been carried out for 1 h at room temperature resulting in the expression plasmid pMAL3-W-C which was transformed directly into the heat shock competent cells *E. coli* XL-10. Different aliquots were spread on 100 µg/ml Ampicillin selective LB-agar plates and incubated at 37°C overnight. The newly constructed plasmid was confirmed with restriction analysis using enzyme *Eco*RI and *Eco*RV before transferring into the expression strain *E. coli* BL21 (DE3). To analyze the fusion gene expression, 5 ml of the supernatant fraction of the expression *E. coli* BL21 harbouring the vector construct pMAL3-W-*C* under specific stress condition as mentioned before, 10 µg of the purified protein of affinity chromatography amylase fractions were loaded on 12% SDS-PAGE gel. The expressed and purified band corresponded to the expected fusion tagged protein (MBP-GlgC) size of ~ 84 KDa (Fig. 35). These construct will be used for further pull-down protein: protein interaction experiment to establish the interaction between the proteins native GlgD from the *Lb. plantarum* WCFS1 strain and fusion MBP-GlgC complex.



a



b

Fig. 35 a: Schematic showning the disruption of the gene *glgD*_his from the plasmid construct pMAL-WCFS1-*glgCD*_his using enzymes *Xba*I and *Sac*II then modification the DNA ends and re-ligation to result new expression fusion vector pMAL3-W-*C*. b2: SDS-PAGE analysis of the expression constructs pMAL3-W-*C* in *E.coli* BL21 under stress condition purified on affinity amylase resin eluted with 100 mM Maltose (E1-3).

3.1.7.3.1 Pull-Down Proteins interaction using the amylase resin column

In a pull-down assay, the protein partner is expressed as fusion protein MBP-GlgC (Bait protein) in *E. coli* BL21 and then immobilized using an affinity amylase ligand resin specific for the fusion tag Maltose binding protein (MBP) as described in this work. The immobilized bait protein was incubated with the lysate of the wild type *Lb. planatrum* WCFS1 which was incubated for different time points of 4, 6, 10, 24 and 48 h with extra amount of 20% glucose-lactose to increase the accumulation possibility of glycogen as well as the expression of the native *glg* genes especially protein *GlgD* which will be used as a prey protein. The entire complex has been eluted from the affinity amylase resin with column buffer supplemented with 100mM Maltose. Two controls kept in parallel for the bait and the prey proteins were analyzed under the same condition. Successfully, the interesting interactions between the proteins GlgC and native GlgD was confirmed and detected by SDS-PAGE analysis. The schematic diagram of the pull-down experiment process of the protein: protein interaction and the SDS –PAGE analysis of the complex protein GlgCD from strain *Lb. plantarum* WCFS1 are shown in Fig.36

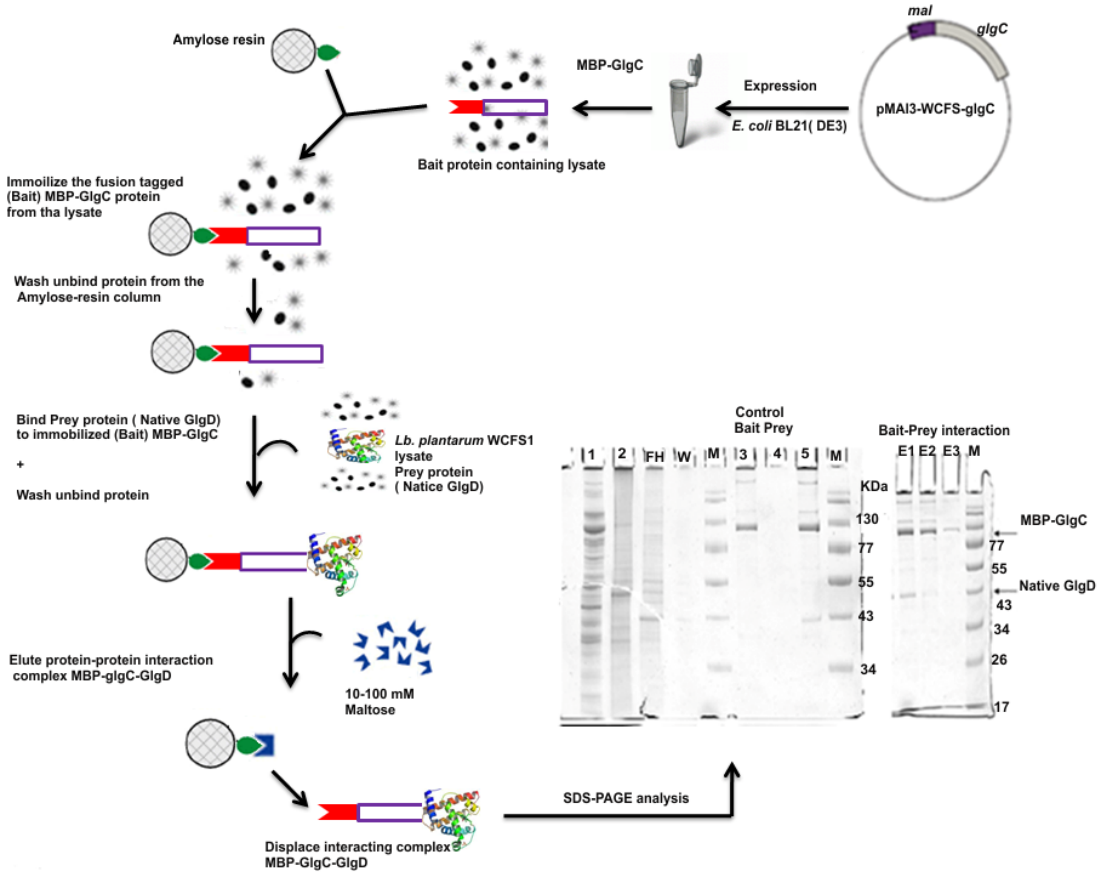


Fig. 36. Schematic outline of Pull-Down assay strategy was used to establish the protein: protein interaction between GlgC and native GlgD. The fusion tagged protein MBP-GlgC (as a Bait protein) expressed in *E. coli* BL21then immobilized using affinity amylase resin and incubate with the supernatant fraction cell free of wild type *Lb. plantarum* WCFS1 with extra glucose at different time point for Prey protein (native GlgD). SDS-PAGE analysis of the pull-Down protein: protein interaction assay. 1: *E. coli* BL21 supernatant pMAI3-WCFS1-*glgC*; 2: Supernatant of Lb.plantarum WCFS1; FH: Flow through; W: wash; M: protein Ladder; lane 3: control Bait protein without prey; lane 4 control Prey without Bait; lane 5: the Bait and Prey sample load and purification together after incubation for 2 hours then washed and eluted as standard protocol with 100 mM Maltose.; E1-3 the elution fraction of Bait and Prey protein together in separate experiment.

3.1.8 Heterologous expression *glgC and glgD* genes in *Corynebacterium glutamicum* with pWLQ2

As mentioned above in *E. coli* BL21, production of the target genes synthesized was hampered by formation of intracellular inclusion bodies aggregates. It may be that the reason of inclusion bodies accumulation is associated to codon usage; the presence of unfavoured codons may repress protein accumulation. Thus, *C. glutamicum*, a Gram-positive bacterium with high G+C content, has been used for commercial production of amino acids, glutamate and lysine and developed as an expression system with same advantage of scalability and ease of culturing (Brinkrolf *et al*., 2010).

Results

Successfully, *C. glutamicum* has been used to express many of heterologous genes. In this work, attempts for heterologous expression of the target genes *glgC* and *glgD* from *L. lactis* subsp. *cremoris* SK11, MG1363 and *Lb. plantarum* WCFS1 species were made using the expression vector pWLQ2. The whole *glg* genes together with RBS were amplified with primer glg-Bam.F/glg-Sal.R or glg.F.Fus/glg.R.Fus using the construct pET101-SK/MG/WCFS1- *glgC, glgD* and *glgCD* as a DNA template. The purified PCR products were cut out with *Bam*HI and *Sal*I and cloned into the shuttle expression plasmid vector pWLQ2 opened with the same enzymes. The resulting constructs pWLQ-SK11/MG/WCFS1-*glgC*/*D*/*CD*-$_{his}$ were transferred into *E. coli* XL1 blue, analyzed by restriction enzymes and DNA sequencing. The correct constructs were transformed by electroporation into the restriction deficient *C. glutamicum* R163. The plasmid isolated from the latter was transformed by electroporation into the lysine-producing *C. glutamicum (*DSM 20300t), the empty plasmid pWLQ2 was also transformed into both the strains in order to obtain a control for expression experiments. The cell was grown on selective overnight growth medium in order to maintain the plasmid. The plasmids were confirmed by isolation (mini prep, QIAGEN) and restriction analyses with enzymes *Bgl*II and *Pvu*I. To analyze the expression of the genes, 5 ml of the optimal growth medium was inoculated with overnight pre-culture with selective antibiotic and incubated until the cells reached the exponential phase of 0.6- 0.8 at OD_{600}. The expressions of the genes were induced with IPTG to a final concentration of 0.1 mM. The incubation at the optimal temperature of 37°C was stopped after 4 h and overnight. The cells were collected by centrifugation at 8000 rpm for 5 min at 4°C and the pellets were resuspended in 50 mM Tris-HCl buffer pH 8 and lysed with ultra-sonication. The crude extracts, supernatants and pellet fractions were analyzed with SDS-PAGE. 10 µg proteins were run on 12% SDS-PAGE gel and electrophoresed at 15 mA. Protein bands were developed using coomassie staining (Fig.38). In all expression fractions, there were no difference in protein intensity bands detected in supernatant as well as in pellet fractions between the positive vector constructs and the controls.

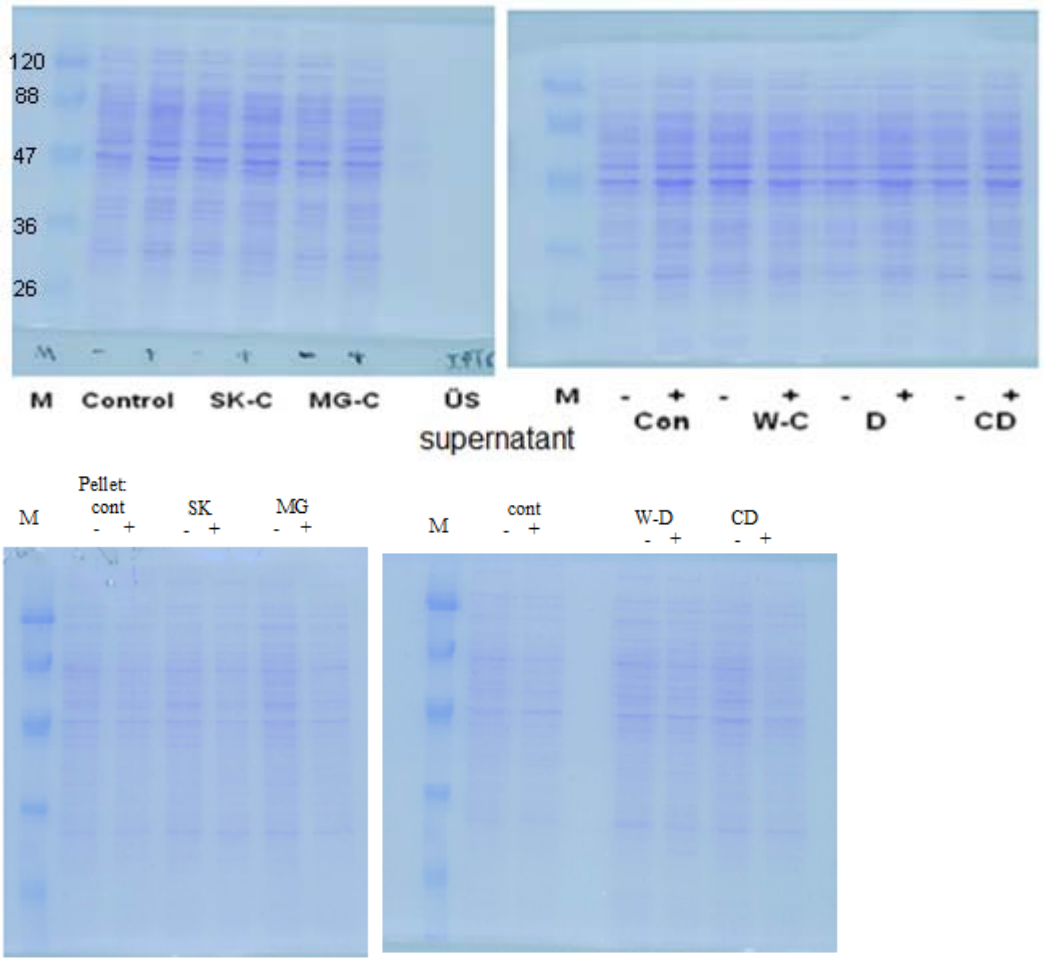


Fig.38 SDS PAGE gels showing the expression of the interest genes *glgC* and *glgD* from *Lb. plantarum* WCFS1 in *C. glutamicum* under the control of the expression pWLQ2 vector [+] and [-] IPTG: SK; pWLQ-SK11-*glgC*; pWLQ-MG-*glgC* and pWLQ-WCFS1-*glgC*/*D*/*CD*_his; Protein Marker (M) [+] and [-] IPTG

3.1.9 **Hetero-expression genes in *Bacillus subtilis***

3.1.9.1 Expression system pHMC02.04.05

The *Lb. plantarum* WCFS1 genes *glgC* and *glgC-glgD* was cloned into shuttle expression plasmid vector pHCMC05. Amplicons from *glgC* or both *glgCD* were generated using primer F.pHC05_Bam and R.PHC05_Xba introducing the restriction enzyme sites *Bam*HI and *Xba*I, respectively, for the ease of cloning in the correct orientation. The PCR was carried out using 64°C annealing temperature with an elongation time of 1 min with fusion polymerase and linearized pET101-WCFS1-*glgC*/*CD*_his as a DNA template. The purified PCR products on 0.8% agarose gel were digested with enzymes *Bam*HI and *Xba*I and cloned into the linearized vector with the same enzymes. The resulting vector constructs pH05-WCFS1-*glgC*/*CD*_his were transferred into *E. coli* XL1 blue, analyzed with restriction enzymes *Eco*RV, *Bgl*II and

*Eco*RI, The correct construct was then transformed into natural competent cells *B. subtilis* 168. The plasmid construct was re-isolated by the alkaline lysis method from *B. subtilis* cells that has been grown in selective chloramphenicol LB growth medium in order to maintain the plasmid. The plasmid was confirmed by restriction analyses with *Eco*RI. The alkaline lysis method gave low plasmid concentration which could not be visualized on the gels. Thus, the same construct was re-transformed into *E. coli* XL1 blue to get huge amounts of plasmids which were isolated and analyzed with restriction enzymes. The empty plasmid pHCM05 was also transformed into the strain in order to obtain a control for the expression experiments.

To analyse the overproduction of the protein in *B. subtilis* 168 carrying the correct construct pH05-WCFS1-*glgC*_$_{his}$, an overnight *B. subtilis* culture harboring construct pH05-WCFS1-*glgC*_$_{his}$ was used to inoculate 30 ml chloramphenicol added LB medium and induced for different times of 1, 2, 3, 4 h and overnight with different final concentration of 0.1 M IPTG; 0.1 or 0.5 mM, when the cells growth reached the log-phase at final optical density at 600 nm (OD_{600} = 0.6-0.7). The cells were harvested by centrifugation at 13,000 rpm for 5 min at 4°C. The pellets were re-suspended in 50mM Tris-HCl buffer pH 8 and lysed with ultra-sonication for 20 min on ice at 30 amplitude and 0.5 cycles. The cell debris was removed by centrifugation at maximal speed of 13,000 rpm for 15-20 min at 4°C. Samples of the crude extracts, supernatants and pellet fractions were analyzed with SDS-PAGE and the proteins band were visualized with fresh coomassie stains for 30 min or used staining solution overnight. No overproduction of the protein GlgC_$_{his}$ was detected at time points 1, 2, 3 and 4 h but increment in a band intensity was observed for overnight expression at induced supernatant fraction level. However, the western blot analysis did not detect any anti-His signal for that band (Fig.39).

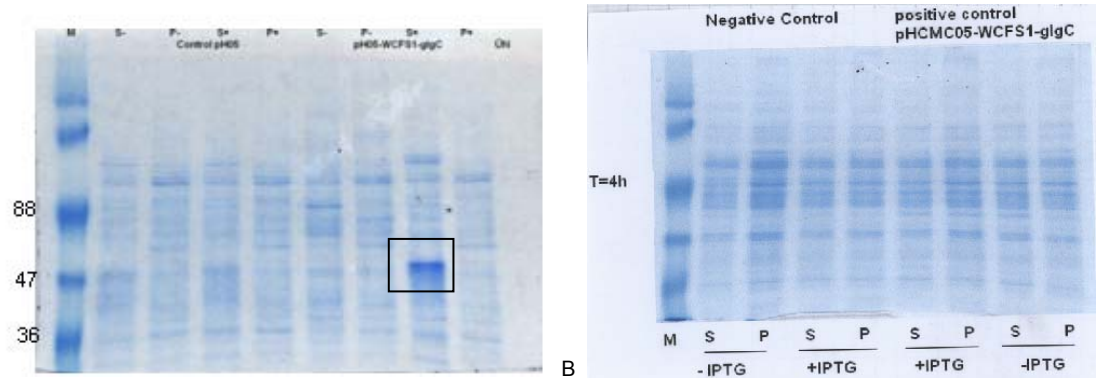


Fig.39. A; SDS-PAGE gel showing the overexpression of the gene *glgC* in *B. subtilis* 168 (pH05-WCFS1-*glgC*_$_{his}$) induced with IPTG and incubate at 37°C for 1, 2,3, and 4 h (B) or overnight (A) . Protein band was shown in the black box in the supernatant fraction comparing with the controls empty plasmid and un-induced. Protein Marker (M) Pellet (P) [+] and [-] IPTG. Supernatant (S) [+] and [-] IPTG

Cloning of both the overlapping genes *glgC* and *glgD* together was done with 6x histidine linker for the ease of purification and western blot analysis. Correct construct purified from *E. coli* XL1 and analysed with restriction enzyme as described above, was transformed into natural competent cells *B. subtilis* 168. The construct pH05-WCFS1-*glgCD*_his was re-purified from ampicillin-resistant *B. subtilis* colonies and analysed with different restriction enzymes *Bgl*II, *Eco*RI, *Eco*RV and *Sac*I before overexpression step. The construct was compared with pHCMC05 digested with the same restriction enzymes. The restriction analyses showed that a huge fragment of the insert *glgCD*_his was missing leaving only around 500bp in the construct (Fig. 40).

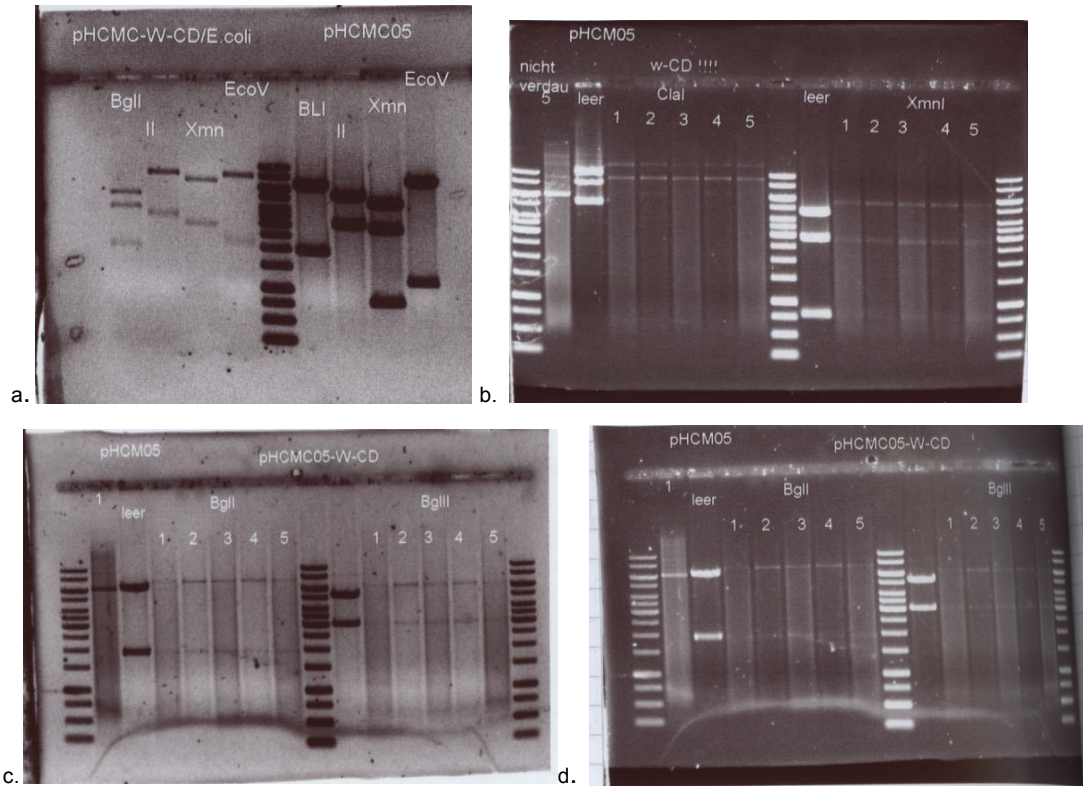


Fig. 40. Restriction analysis of the cloning the gene *glgC* and *glgD* from *Lb. plantarum* WCFS1 in the expression vector pHCMC05. A; confirm the correct construct in *E. coli* XL1 before transfer into *B .subtilis.* B, C and D*;* shown the restriction analyse of the construct purified from *B .subtilis* with different restriction enzymes, the First line is empty vector pHCMC05 was used as a control for comparing. Lane 1-5 the isolated expected construct from *B. subtilis* showns the missing of fragment of the insert *glgCD* from the purified vector pHCMC05-W-*glgCD* after transferred the correct construct from *E. coli* XL1 into *B. Subtilis.*

3.2 Functional Analysis of the genes *glgC* and *glgD* by gene knockout

Several strategies were employed for the Inactivation/knocking out of the *glgC* and *glgD* genes of *Lactococcus lactis* subsp*. cremoris* MG1363 and *Lactobacillus plantarum* WCFS1.

3.2.1 **Single crossover.**

A- Integration plasmid was derived from the Gram-negative bacteria cloning pSC-A plasmid. To construct a single-crossover disruption mutant of *glgC* and *glgD* genes, internal gene fragments of approximately 700 bp from *L. lactis* and *Lb. plantarum* were generated by PCR. *Eco*RI enzyme cloning site was introduced to the used primers WCFS1-C-Knock.F2, WCFS1-C-Knock.R2, WCFS1-D-Knock.F2 and WCFS1-D-Knock.R2 (Table 2: primer list, material and methods). *Taq* DNA polymerase was used to amplify the fragment. The PCR products were purified from 0.8% agarose gel and digested with enzyme *Eco*RI before cloning into the dephosphorylated ends of purified plasmid pSC-ΔC1/D1 that was linearized with the same enzyme. The antibiotic Erythromycin cassette (Em) was amplified from the cloning plasmid pMAD2 using primers F.Erm and R.Erm with specific overhangs of enzyme sites for *Bam*HI and *Xba*I respectively, and cloned into the vector construct resulting in the pSC-MG/W-Δ*C*1/*D*1-Em knock-out vector. Despite several attempts, no positive erythromycin-resistant (Ery^R) colonies were obtained from transformation *Lb. plantarum* WCFS1. The reason may be attributed to the low transformation efficiency of the competent cells prepared by different methods. In case of *L. lactis* MG1363 transformation, a few erythromycin-resistant colonies had been obtained. The purified fragment of 700bp *glgC*1/*D*1 PCR product was used as a probe for southern blot analysis after labelling with biotin (material and methods section 2. 6 Southern blot and labelling). The protocol for the preparation of *L. lactis* MG1363 competent cells was optimized and the transformation efficiency was identified using the cloning shuttle plasmid *E. coli*/lactic acid bacteria pTRKH3 (BCCM/ LMBP, Belgium). PCR and chromosomal southern hybridization analysis did not show any positive gene knock outs from the colonies tested.

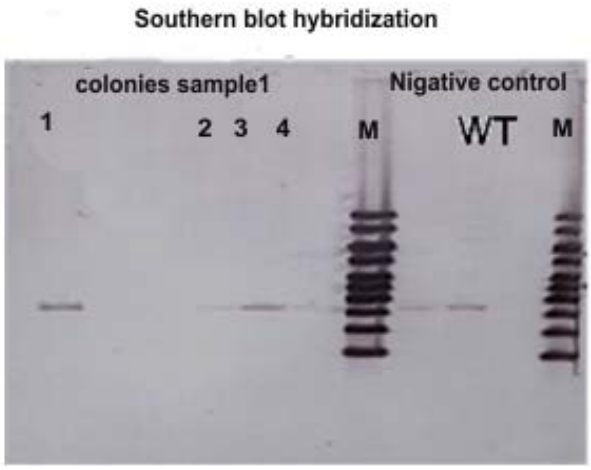


Fig. 41. The southern hybridization of chromosomal erythromycin-resistance transforming colonies *L. lactis* with pSC-MG::*C*1; 1, 2, 3, 4 Ery^R colonies

B- Bacterial conjugation is a common mechanism for genetic exchange in nature. In this attempt, disruption of the target genes by single crossover replacement was based on the conjugation transfer properties of the plasmid of *Lactococcus* strain. A new plasmid has been derived from the conjugated plasmid pK19mobsacB which was successfully used to transfer and disrupt the genes in Gram-positive bacteria.
The *glgC*1/*D*1-*Erm* fragments were obtained from the digestion of the vector pSC-M-*C*1/*D*1-*Em* with restriction enzymes *Hin*dIII and *Eco*RI and cloned into the linearized plasmid pK19mobsacB with the same enzymes resulting in the new conjugated vector pK19-M-*C*1/*D*1-*Em*. Few erythromycin-resistant (Ery^R) colonies were obtained. Chromosomal southern blot analysis showed that transformant colonies had the same pattern of the wild type hybridisation. No integration was detected in either of the tested colonies. Colonies were grown under selective condition in optimal MRS broth to analyze vector integration and/or exclude replication possibility of the plasmid. Recombinant plasmid DNA was isolated from 5 ml overnight culture of the plasmid free strain *L. lactis* MG1363 (Material and methods section 2. 4, plasmid purification). Poor plasmid yield from lactic acid bacteria resulted in difficult visualization of the digested plasmid concentration. The purified plasmid was re-transformed into *E. coli* XL1 and analyzed with restriction enzymes *Bam*HI and *Eco*RI. Purification of the plasmid from the transformants indicates that the conjugate knockout vector was able to replicate in the bacteria *L. lactis* MG1363 with an unknown mechanism. Furthermore, about a 2 Kb fragment was missing from this isolated plasmid compared with the expected transferred conjugated DNA plasmid (Fig 42).

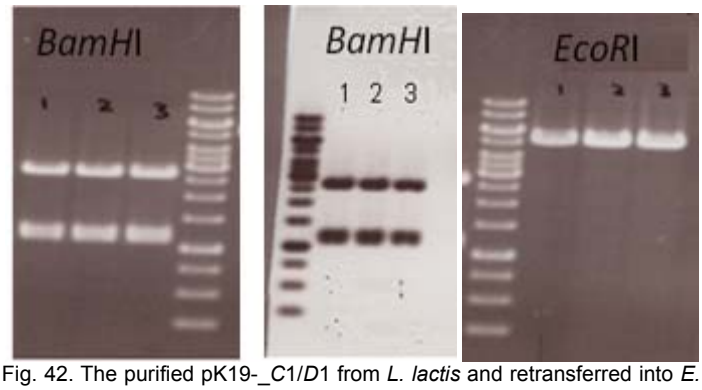


Fig. 42. The purified pK19-_C1/*D*1 from *L. lactis* and retransferred into *E. coli* XL1 was digested with *Bam*HI, *Eco*RI for 1 h and electrophorssis on 0.8% agarose gel for 40 min. The bands show smaller size than the expected size of conjugated vector and/or basic plasmid approximately 3 Kb was missed. *Bam*HI cut one time in the basic plasmid pK19mobsacB (5.7Kb). The expected size for *Bam*HI and *Eco*RI in vector pK19-M-*C*1/*D*1-*Em* ~6.5+1.17 Kb and 5.7+1.2+0.7Kb respectively

C- Based on the cloning vector pBluescript SK, a new single crossover replacement vector was constructed in an attempt to disrupt the target genes *glgC* and

glgD in *L. lactis* MG1363.

About 700bp fragment of the gene *glgC/D* of *L. lactis* MG1363 has been amplified using the primers F.M.C1 and reverse primer R.M.C1 with *Eco*RI overhang. Both the PCR product and plasmid pBluescript SK were digested with the same enzyme. The digested plasmid was purified and dephosphorylated to decrease the self-religation frequency. Cloning of the PCR product resulted in the plasmid DNA pBlu-M-*C*1. The Erythromycin cassette was amplified from shuttle cloning plasmid pTRKH3 using primers F/R. Ery and cloned to pBlu-M-*C*1 at the restriction site *Bam*HI and *Xba*I resulting in the knockout vector pBlu-M-*C*1-*Em*. The final construct was analyzed with different restriction enzymes to confirm the correct target construct. The Ery^R colonies were selective on MRS selective medium with a final concentration of 5µg/ml of Erythromycin. Southern hybridization chromosomal blot and PCR analysis were performed to confirm the gene knock out with different primers and wild type genomic DNA of *L. lactis* was used as control. No mutant was detected from either of the tested colonies.

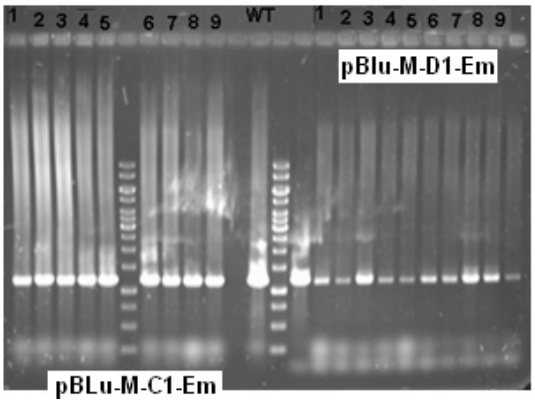


Fig.43. The PCR analysis of the erythromycin-resistance colonies were grown after transformed the pBlue.M.*C*1/*D*1-*em* shown same size of the wild type strain *L.lactis* MG1363. 1-9 numbers of colonies were analysed

3.2.2 Insertional inactivation genes *glgC* and *glgD* by Single/Double crossover using pME-1 new knockout derived from cloning plasmid pS72 (Promega).

To generate new stable knock out plasmid in *L. lactis*, the plasmid pME1 (provided by Prof. Dr. Vogel Lab, TU-München, Germany) that used successfully to knock out genes in Lb. Sanfraciscensis.

Fragment of the gene *glgC* was obtained from PCR using primers F.knock.C1and R.Knock.C1, (Table 2, material and methods) carrying the *Eco*RI restriction site.

About 500bp fragment of the gene *glgD* along with the upstream *glgC* were amplified using the proofreading *pfu* DNA polymerase to obtain a 1.7 kb fragment for increasing

the homologous sequence and the integration ratio. Primers F.M.C2_Xho and R.M.D1_Hind were used. Digestion and ligation into the restricted plasmid pME1 with the same enzymes resulted in the nonreplicating integrated single crossover plasmids pME1-M-*C*1 and pME1-M-*D*1, respectively, which were transformed into *E. coli* XL10 cells. The constructs were purified and confirmed with restriction enzymes before electroporation into the electro-competent *L. lactis* MG1363 cells .The expected mutant Ery^R colonies were analysed by the iodine phenotype test (Fig. 44) and the genotype analysed with PCR and southern hybridization blot.

The Ery^R colonies (pME1-M-*D*1) have shown the positive phenotypes of light brown colour compared with the brown colour of the glycogen accumulation in the wild type *L. lactis* MG1363. The colonies were cultured under selective control in MRS broth and the genomic DNA was purified from the overnight culture for further analysis. No integrations in the chromosomal genome were detected with PCR analysis. Preceding efforts to inactivate the gene *glgC* using different single-crossover knockout vector were abandoned after such unsuccessful attempts

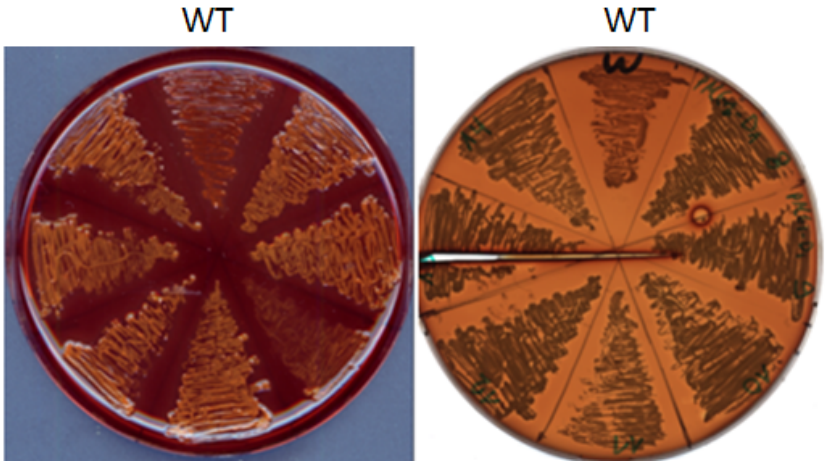


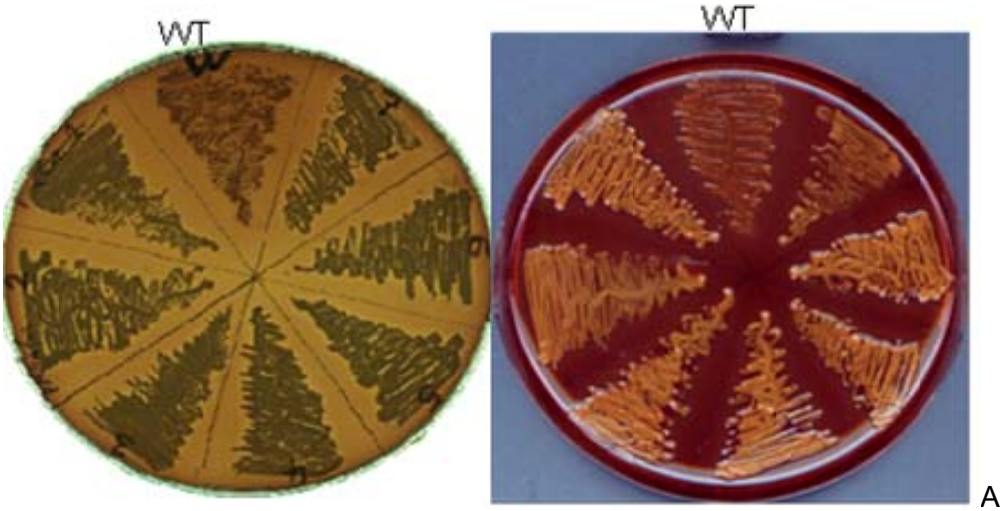
Fig. 44; The iodine test of transformants pME1-M-*D*1 Ery^R colonies was shown a light Braun colour compared to the Braun colour of accumulation glycogen in the *L. lactis* MG1363 wild type

A new strategy was adopted by which double crossover knockout vector was generated using two 500 bp fragment C1 and C2. Each of the targets were amplified form the gene *glgC* by the primers F.M.C1_Xho, R.C3_hind, F.M.C2a_Bam and R.SK11, respectively introducing specific restriction enzyme sites. A two step cloning was followed of which the first one involve digestion and ligation of the C1 fragment into the plasmid linearized with enzyme *Xho*I and *Hin*dIII resulting in pME-M-*C*1a, which was transformed and purified *E.coli* XL1. The construct pME-M-*C*1a was analyzed and digested, for the second cloning step in which it was digested with the enzymes *Bam*HI and *Eco*RV and purified *Bam*HI digested PCR fragment C2 was

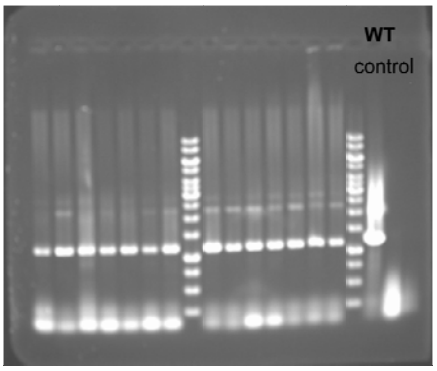
cloned at the same restriction site. The resulting double crossover integrated plasmid pME2-M-*C*2 was transformed and analyzed from *E. coli* XL10 before electro-transformation into *L. lactis* MG1363.

After transferring the double-crossover vector pME2-M-C2, a few of the Ery^R colonies were grown (no colonies were obtained in further repeating transformation step). The colonies appeared creamish in colour thereby showing the positive phenotype compared with the brown colour which could have appeared due to the accumulation of the glycogen in the wild type *L. lactis* MG1363(Fig. 45A). Otherwise, the genotype analyses of the Ery^R colonies by PCR have not detected any positive stable mutant (Fig. 45B) two bands were obtained the 1.2 Kb similar to the wild type expecting size and the other band 2.4 is corresponded to expect size of the double crossover integration plasmid.

These results may indicate unstable plasmid integration into the diploid chromosomal DNA of the *L. lactis* subsp. *cremoris* MG1363. Further experiments are required including modification transformation protocol to increase the transformation efficiency.



A



B

Fig.45 A; The Iodine test of transformants pME2-M-*C*2 Ery^R colonies was shown a creaming colour compared to the wild type (Braun colour refers to glycogen accumulate). B: PCR analysis of the genomic DNA of the transforming pME1-M-*D*1 and pME2-M-*C*2 Ery^R colonies. The expected size in wild type about 1.2 Kb and in positive double crossover integration about 2.4 Kb

3.3 Homologous Expression the *glg* genes in *Lactococcus lactis*

3.3.1 Expression the genes under NICE (Nisin Controlled gene Expression) system

This system is used for the expression of homologous as well as heterologous proteins in lactic acid bacteria. Such expression for *L. latis* subsp. *cremoris* MG1363 genes was carried out using popular vector for nisin-inducible expression, pMSMP3535, which was kindly supplied by Dr. Gary M. Dunny, University of Minnesota (Edward M. Bryan *et al*., 2000).

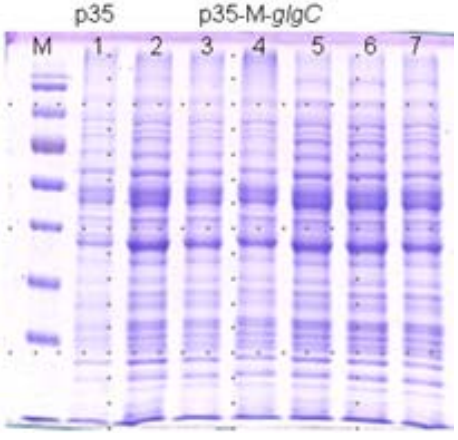
For overexpression, target genes *glgC* and *glgD* from *L. lactis* MG1363 and ORF1 (174434-172767-ncontig) ORF2 (45942-49364+ alsaactr F02. amino acid sequencing NCBI blast: dextran-glucanosucrase- denoted as GLu hereafter) and ORF3 (56748-568580+ncontig/371aa NCBI blast glucosidase enzyme -denoted as glcA hereafter with small domain 58aa upstream of this ORF) from *Lb. sanfranciscensis.*

The aforesaid genes were cloned into the plasmid pMSP3535 in a two step process. The first them was by amplifying the target gene by PCR using special primers (60 °C and 70°C annealing temperature for *Lb. sanfraciscensis* and *L. lactis* genes, respectively) and purifying the PCR products which were subcloned into the cloning plasmid pJET. In the next step, the fragment of interest was excised by *Bam*H1 and *Xba*I digestion to obtain the restriction site in a correct orientation of N-C terminus of the target gene and re-cloned into NICE plasmid pMSP3535 linearized with the same enzymes. In the resulting constructs designated as p35-M/San-target gene, the target gene under control of the promoter P*nisA* and its expression could be induced with nanogram (ng) of Nisin. The vector was isolated from *E.coli* XL10 positive clones selective on 250 µg/ml erythromycin and was restriction analyzed and correct constructs were confirmed by sequencing the DNA before electroporation into competent cells *L. lactis* subsp. *cremoris* MG1363 The empty plasmid pMSP3535 was also transformed in order to obtain control strain for further expression experiments. All constructed strains were grown in MRS medium supplemented with erythromycin to a final concentration of 5 µg/ml in order to maintain the vector.

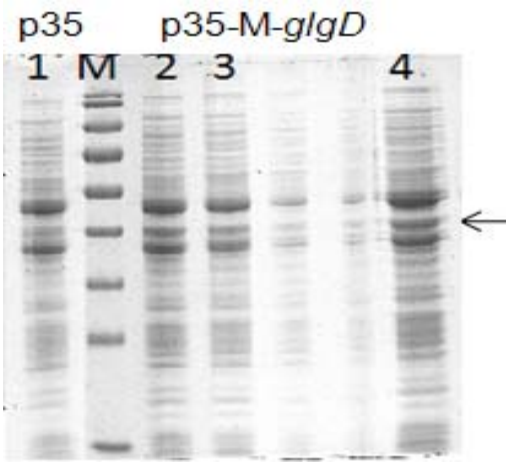
Analyses of the homologous and heterologous expression of the *glgC*/*D* genes (*L. lactis*) and *ORF*s (*Lb. sanfraciscensis*)

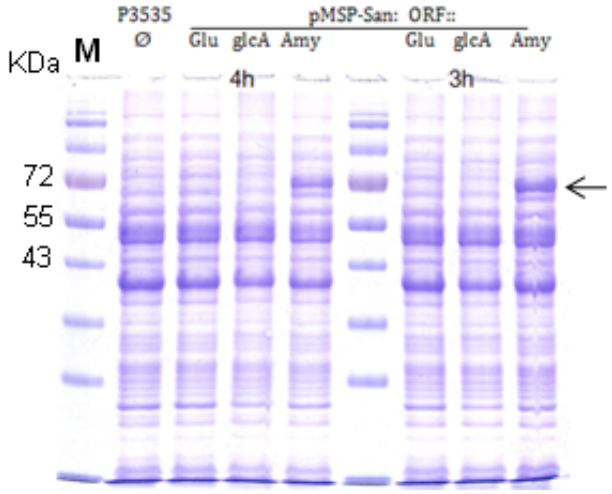
The *L. lactis* subsp. *cremoris* MG1363 strains carrying the p35-M/San-target gene or control pMSP3535 plasmids have been grown at 30°C without shaking in 5 ml MRS medium supplemented with erythromycin. The pre-culture was used to inoculate 10, 30 or 500 ml culture at a starting OD_{600} of 0.05-0.1. The cells were grown to an early log-phase of 0.4 at OD_{600} and the target gene expression was induced by adding Nisin (Sigma, Germany) to final concentrations of 0.1, 1, 3, 5 and 10ng/ml in the medium. The incubation at 30°C was stopped after 3-4 hours of induction and the cells were harvested by centrifugation at 4°C (for 5-20 min depending on culture volume small or large, respectively). The cell pellets were resuspended in suspension buffer 20 mM Tris-HCl pH 7- 8 and opened by sonication or french press corresponding to the sample volume. The cell debris was removed by centrifugation at 13,000 rpm in case of small culture volume while at 21000 rpm for 20 minutes in case of 0.5 liter culture. The supernatants, crude extracts and pellet fractions were analyzed on 12% (w/v) SDS-PAGE gel and the proteins were visualized using coomassie staining (Fig. 46 A and C). These results show no increase in the intensity of expected protein bands of the genes *glgC*, *ORF*2 and *ORF* 3. Thus, expression could not be detected and there was no difference between the positive construct and the control that expressed under same condition. On the other hand, an interestingly increase in the intensity of protein bands were detected in the expression of the genes *glgD* and *ORF*1 (*Amy*) compared with the empty vector control under the same condition (Fig. 46 B and C).

a.



b.





c.

Fig.46. SDS-PAGE gel showing the homo-heterologous- expression of the proteins in cells supernatant fraction under the NICE system using the PMSP3535 with different nisin final concentration 0.1, 3, 5, 10 and 20 ng/ml,The arrow indicates to the increasing intensity of the expecting protein bands of GlgD and ORF 1(Amy) proteins respectively.

a- Expression the construct pMSP3535-MG-*glgC* (p35-M-*glgC*)

b- Expressin the construct pMSP3535-MG-*glgD* (p35-M-*glgD*)

c- Expression the constructs pMSP3535.San-Target ORf(Glu, glcA amd Amy) for 3 and 4 h.

It might be so that our result corresponds with the reason previously proposed by Kim and Mills (2007) for a non expression or too low expression level. They assumed that this version of pMSP3535 missed a transcriptional terminator which probably leads to the disruption of the expression of the *nisK* and *nisR*, the essential genes of the NICE expression system. Hence, the non-stop transcription reading from the powerful *nisA* promoter of the two component-inducing genes in an opposite orientation leads to the case of successful expression of *glgD*. This was possible due to the coincidentally amplified 50bp downstream *glgD* gene region which has been shown to have a high probability of the presence of native transcription-terminator predicted by bioinformatics analysis (Fig. 47).

Presence of the native transcription terminator of the genes *glgCD* may explain the successful expression of the GlgD protein as well as ORF1 (Amy).

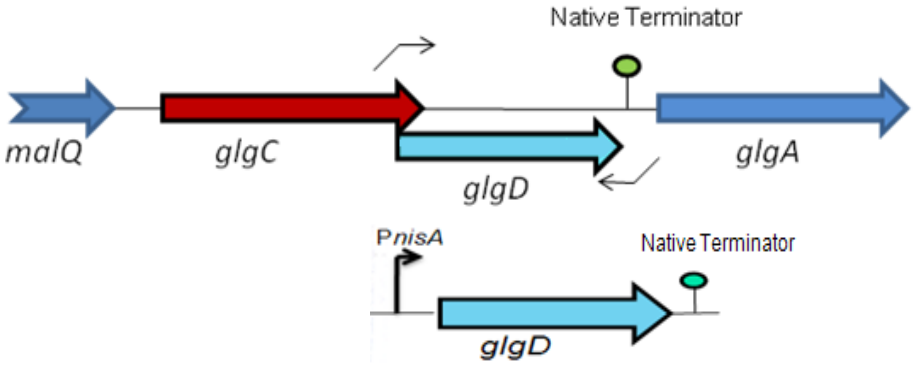


Fig.47. Schematic explains the reason of successfully expression of protein GlgD using the bioinformatics analysis which confirm previously proposed by Kim and Mills (2007).

The previously underestimated absolute copy numbers of the plasmid could be attributed to the total DNA isolation technique or loss of selection pressure in overnight cultures. The influence of pMSP3535 copy number on protein expression by several modification of pMSP3535 has been reported by Kim and Mill (2007). The level of protein expression was improved by, reducing overall expression burden of *nisA* promoter, inserting an independent transcription terminator to prevent possible interruption of the nisin signal transduction and increasing the plasmid copy number. The resulting plasmid pMSP3535H2 showed improvement in protein expression. The nisin immunity genes *nisI* were previously reported to be most important determinant among several immunity genes in the nisin gene cluster (Beerthuyzen *et al*., 1999). Thus, *nisI* was incorporated into the further improved version of the plasmid resulting in pMSP3535H3, which proved to increase nisin tolerance in bacteria hosting it, thereby leading to increase in the recombinant protein production due to higher dose of nisin which can be added into the culture (Gian *et al*., 2009).

The improved plasmid pMSP3535H3 (pH3) (kindly supplied by Dr. David A. Mills, University of California, USA) was used in this work for homologous expression of the genes *glgC* and *glgD* from *L. lactis* subsp. *cremoris* MG1363.

The target genes were cloned into the improved plasmid pH3 after modifying the C-terminus of the genes with poly-His-tag (6xHis). The C-terminal ends of the target genes were modified to facilitate the purification and detection process of the proteins of interest.

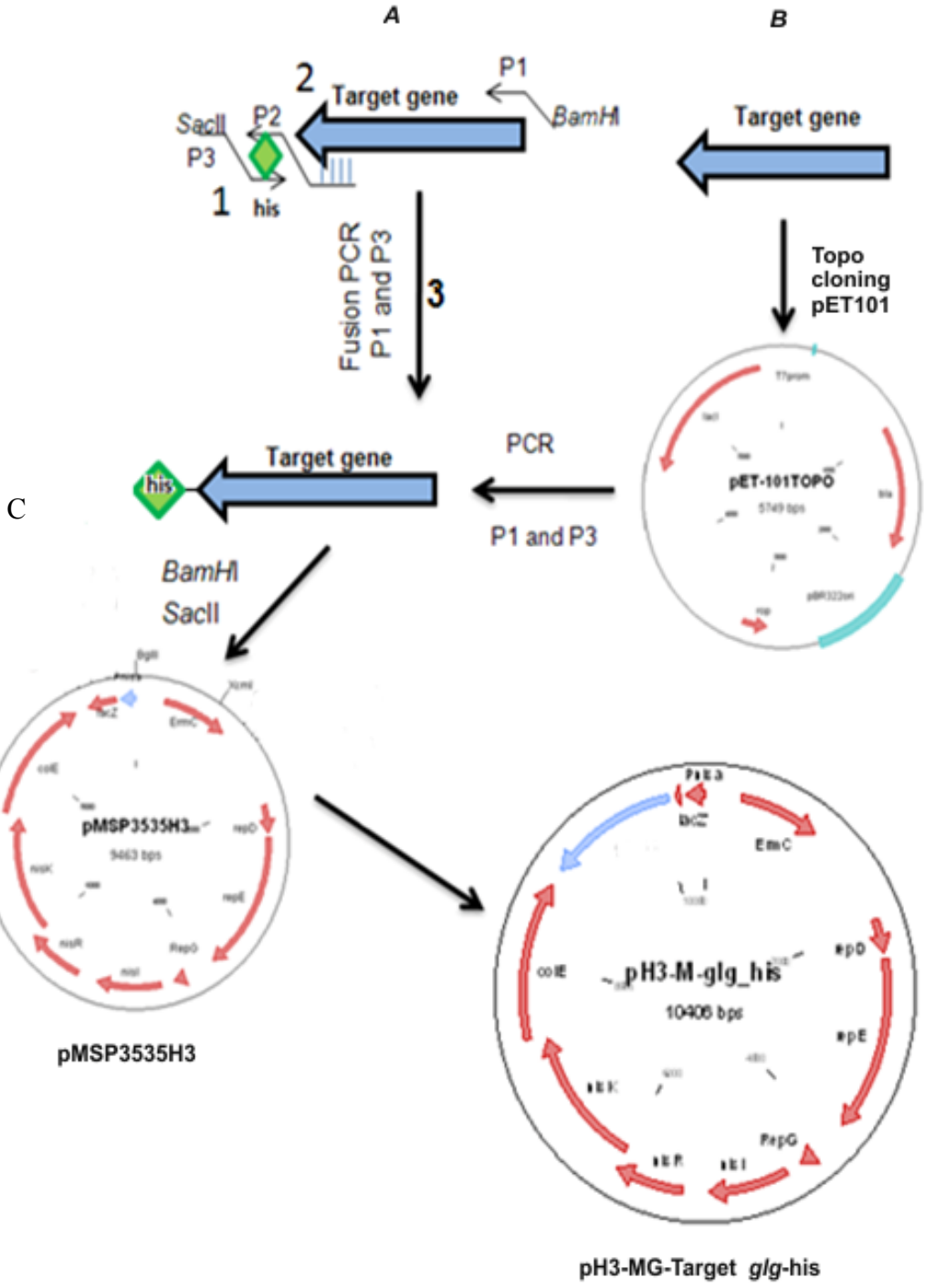


Fig.48. Schematic show the cloning strategies of fusion target gene-His tag (obtained from two strategy A or B) were used to construct new expression vector from improved pMSP3535H3 (pH3) under NICE expression controlled system. A: Linker-Fusion PCR/ ligation the target gene with the polyhistidine. B: sub-cloning target gene to pET101 to obtain the His-Tag linker downstream of the target gene.

Two strategies were generated to add the His-Tag linker to the C-terminus of the target protein.

The first was based on a modified ligation procedure involving the-complicated linker-ligation/fusion PCR reaction: for the introduction of small DNA fragment (carrying His-

Tag) into the target gene DNA sequence. The long DNA primers P2 and P3 were designed to be complimentary to each other for about 25 bases from the 3´end and the 5' end was homologous to the target gene sequence for the primer P2 and the restriction *Sac*I linker for.P3 (section 2. 2. 8, linker ligation, material and methods) (Fig. 48 A).

The second method was based on the sub-cloning of the PCR amplified target gene into the pET101. The total target gene along with the his-tag in the correct orientation from the C-terminal was amplified using the forward primer P1 of the target gene carrying a linker for restriction enzyme *Bam*HI or *Xba*I sites and the reverse primer P3 tagged with His_*Sac*II (Fig *48* B).

The successfully modified genes were cloned in the *lacZ* reporter of the plasmid pH3 at the site *Bam*H1 and *Sac*II. The cloning strategies were focused on expression of each gene from each of the strains to characterize the individual protein functions and in conjunction (Fig. 48 C).

The expression vector construct pH3-MG-*glgC*_$_{his}$, pH3-MG-*glgD*_$_{his}$, pH3-MG-glg*CD*_$_{his}$ and pH3-San-ORF2 *Glucansucrase* (GLu) were identified with restriction analyses and the correct constructs were confirmed by sequencing of the DNA (GATC Biotech).

Mutation free vector DNA constructs were transformed by electroporation into the electro-competent cells *L. lactis* MG1363 with the following parametes1.5 KV, 25 µF and 300 Ohm with a constant time of 4-5 ms in the GenePulser II chamber (Bio-Rad Laboratories, Munich, Germany). Subsequently, 900 ml cold MRS broth was added to electroporated-mixture and incubated on ice for an additional 5-10 min. And then was incubated at 30°C for 2 h. The positive transformants were selected on MRS-Agar medium supplemented with Erythromycin to a final concentration of 5 µg/ml and incubated at 30°C for 2 days.

To analyse the protein expression, a 10 ml MRS broth was inoculated with the overnight selective broth until it reaches the early exponential growth phase (OD_{600}=0.3-0.4). During that growth phase different amounts of nisin e.g. 1, 2, 3, 4, 5, 10, 20, 30 and 40 ng/ml were added to the cell cultures in order to obtain the best level of target protein expression and incubated at the optimal temperature for 3, 4 h and overnight without shaking. The supernatants and crude extract fractions (obtained as described before) were analysed on 12% SDS-PAGE gel. The visualization of the protein bands in gels have shown significant increase in the expression of the proteins in the construct carrying *glgD* alone or with *glgC* compared with the control harbouring the empty plasmid under the same expression conditions, while no expression was

obtained in case of the construct *glgC* and ORF glucansucrase.(Fig. 49)

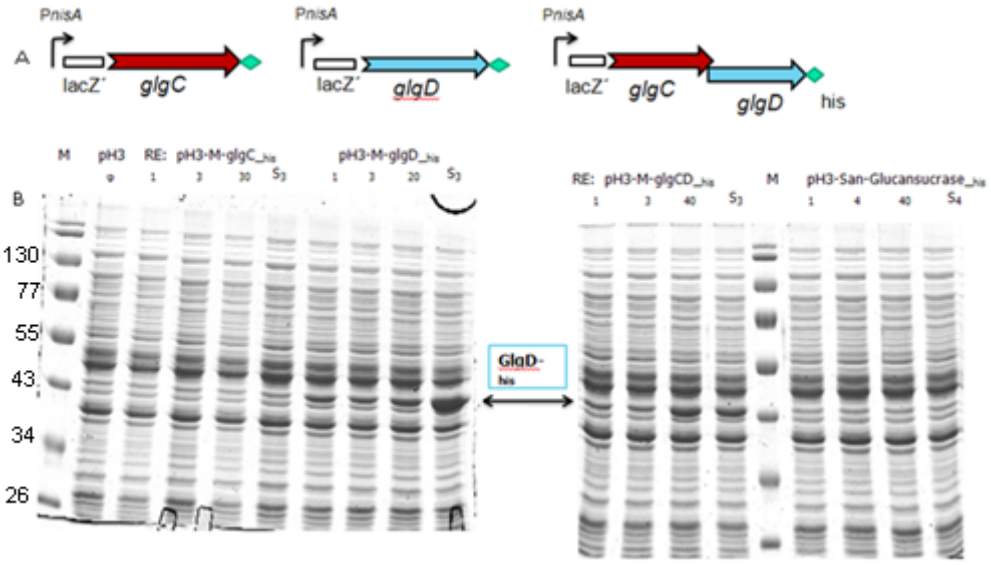


Fig. 49 A; SDS-PAGE gel showing the cloning strategy was used to clone the modified target genes into the improvement nisin system plasmid pH3. B; the SDS-PAGE analysis of the homo-expression genes in *L. lactis* MG1363 under nisin promoter control with different final concentration of nisin 1, 3, 4, 20, 30 and 40 ng /ml for 4 hours induction at 30°C.

The overproduced polyhistidine tagged protein GlgD_$_{his}$ was purified successfully using the Ni-IDA column (Protino) as shown in Fig. 50. The purified fraction of the protein GlgD_$_{his}$ was used for further activity characterization assay.

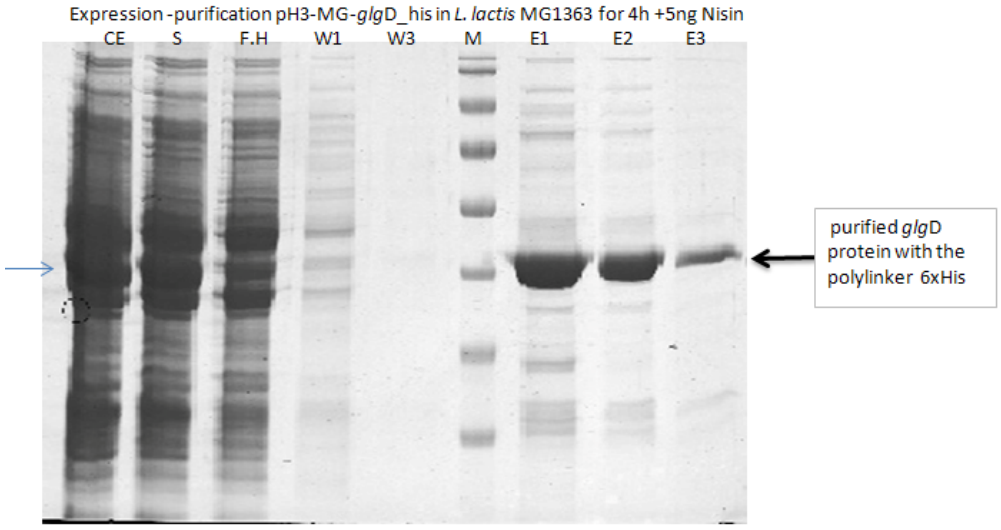


Fig. 50 SDS-PAGe gel showing the purification the overexpression of gene *glgD*_$_{his}$ from supernatant fraction of *L. lactis* MG1363 carrying the pH3-MG-*glgD*_$_{his}$ induced with nisin to final concentration 5 ng/ml for 4 h, the cells harvested and opened resuspended cells with 1xLEW buffer with french press, cells debris collected with centrifugation at 20,000 rpm at 4°C for 20 min and the supernatant fraction purified on the Ni-IDA
S-soluble fraction; CE-crude cellular extract; FH-flow through fraction; W-washing fraction and E-elution fractions

The elution purified fractions of overproduced GlgD protein were concentrated using the Vivaspin 15 ml ultra-filtration devices as recommended by the supplier´s instructions (Sartorius stedim) with 5 KDa cut off. The protein activity was assayed according to the modified colorimetric method for the assay of ADP-glucose pyrophosphorylase (ADPPase) (Fusari *et al*., 2006) (section 2.11.1 material and methods). The reaction was incubated for different time points and the best result was obtained after 10 minutes of incubation. The protein GlgD has shown a slight activity at 630 nm (Fig. 51). The *glgC* gene encoding the ADPPase enzyme in many organisms has been previously proposed to have a homotetramer structure with catalytic and regulator function control producing the essential glycogen subunit ADP-Glucose of glycogen synthesis pathway (Preiss 1984; Ballicora *et al*., 2003; Gerd Seibold *et al.,* 2007). However, in *Bacilli* and plants, the enzymes are heterotetramers consisting of two identical subunits suggesting one to be catalytic and the other presumably responding to allosteric regulation (Hiroki Takata *et al.,* 1997; Nikolaos *et al*., 2007). The amino acid sequence analyses of the GlgC and GlgD proteins show identity of about 24-27 % and up to 40-50% similarity. According to our result in this work and the lack of sufficient information about the function of the GlgD protein as well as the relative homology between the two proteins GlgC and GlgD, two hypotheses has been proposed in the discussion section.

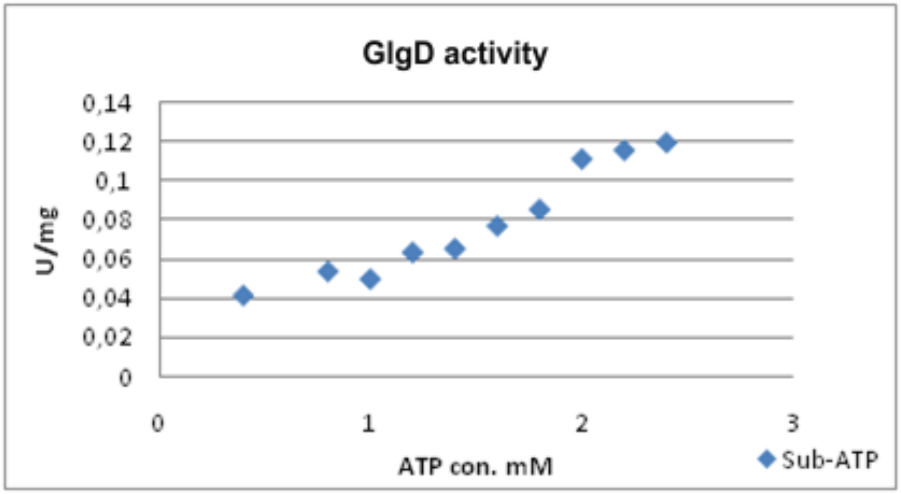


Fig. 51. The activity assay of purified GlgD protein

To exclude the assumption of interaction of the expressed GlgD protein with the native GlgC giving rise to the slight activity, the overexpression is required in the strain devoid of *glgC* gene. This was essential as knocking out of the gene *glgC* in *L. lactis* subsp. *cremoris* MG1363 had been unsuccessful until now. In the current study, thus, using

the free glycogen lactic acid bacteria strain as a host for the heterologous expression from the construct pH3-MG-*glgD*_his_gives the advantage to obtain pur GlgD protein.
The schematic diagram shown below explain the assumption of the slight activity of GlgD obtained by homologous expressed in L.lactis and the alternative strategy was used to exclude the interaction assumed by expression in free glycogen strains or *glgC* knockout *L. lactis* strain (Fig. 52).

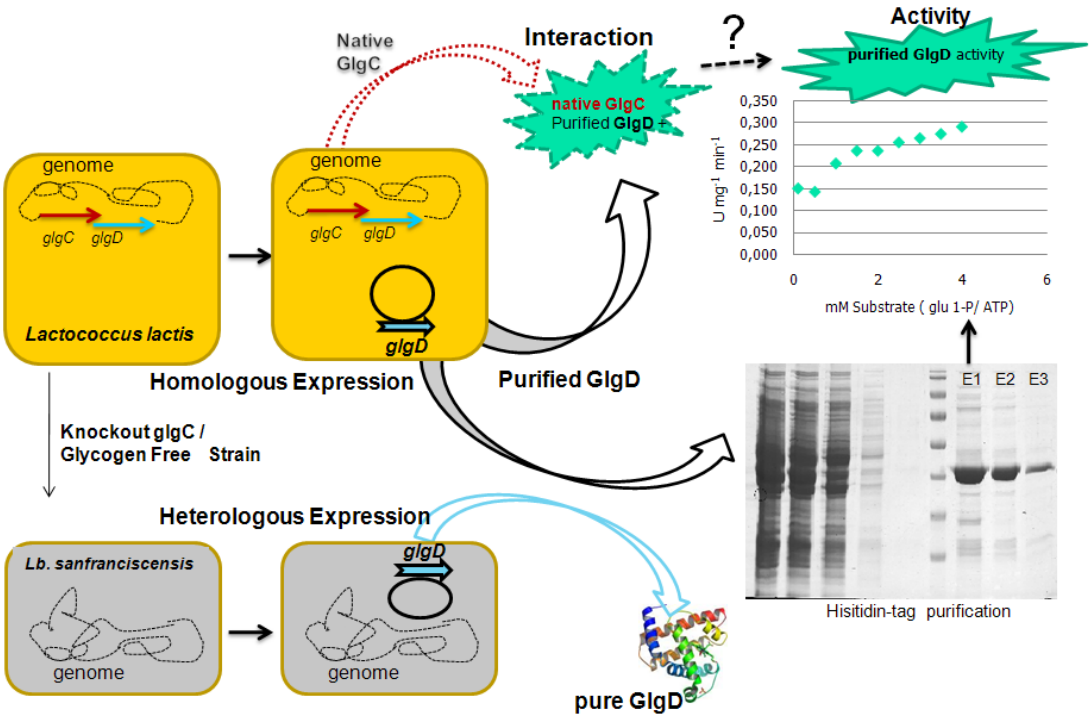


Fig. 52. Schemtaic show explan the strategy plan of slight activty of GlgD protein homologous expression and alternative strategy to exclude the assumption of the reason of the slight activity by expression the construct in free glycogen strain to obtain the pure protein.

Two free glycogen strains have been chosen for these propose namely *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. sanfranciscensis*. Using the latter as a host was an issue due to the limited growth in liquid broth taking either 2-3 days mostly or yielding no growth. The problem with the former as an expression host was, two fold. One was that the growth was optimized by adding skim milk plus 0.5% lactose to the MRS medium and the other was preparing competent cells and plasmid transformation into this strain needed more optimization.

Furthermore, the purified GlgD was shown in this step a high affinity to the substrate UTP than the ATP (Fig. 53). As it is known that the gene *galU* encoding the UDP-Glc-pyrohpoaphorylase (UDPPase) enzyme catalyze the synthesis of UDP-Glucose, the subunit of exopolysaccharide (EPS) synthesis, it is catalyzing a similar reaction of

ADPPase enzyme with UTP as substrate instead of ATP. GalU is 313 aas with (Mol Wt 35001.7) an identity of 20% and about 42% similarities to GlgD protein.

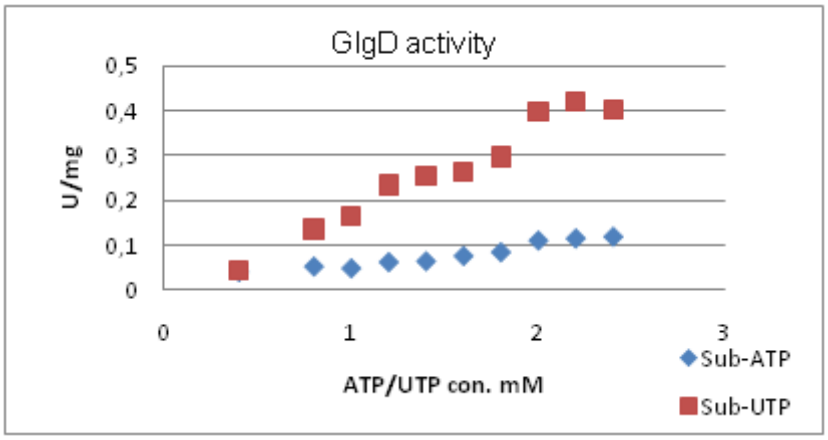


Fig. 53. The affinity of the purified GlgD protein to the substrate ATP and UTP, the protein show higher affinity to the substrate UTP more than ATP.

3.3.2 **Expression attempts of gene *glgC***

As mentioned before, for some unknown reason the gene *glgC* could not be successfully expressed separately in the construct pH3-MG-*glgC*$_{\_his}$ or even when simultaneously amplified together with the gene *glgD* as one insert in the construct pH3-MG-*glgCD*-$_{his}$. The comparative amino acid and DNA sequence analyses at the level of gene insert in both the expression constructs under nisin control promoter pH 3-MG-*D*-$_{his}$ and pH3-MG-*C*-$_{his}$ probably indicate that cloning of the gene *glgC* in the LacZ reporter cassette at the restriction site of the enzymes *Bam*HI and *Sac*II cause disruption of the expression at the level of transcription or translation. It could be so that the stop codon contained in *glgD*, as a result of overlapping with *glgC,* stops the reading frame of the *lacZ* at the beginning of *glgD* which would give the expression for GlgD protein. However, in case of *glgC*, the reading frame of *lacZ* will be continued through *glgC* to stop after 20 amino acids thereby leading to misread the frames to start at methionine of GlgC for expression. Schematic diagram is shown with the assumption of the above explanation for the non expression of the *glgC* gene according to *lac*Z disruption (Fig. 54).

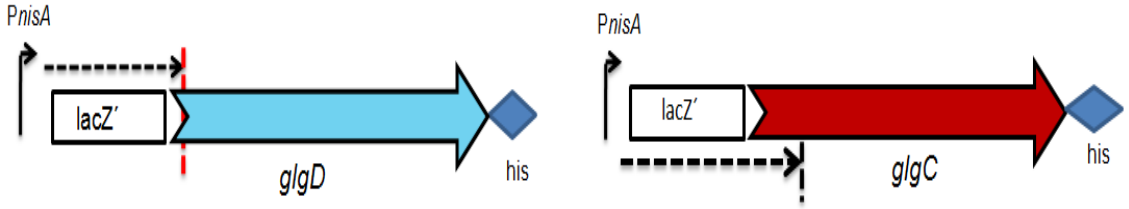


Fig. 54. Schematic diagram showing the assumption of the *LacZ* expression effect in the cloning of *glgD* and *glgC* which was thought to be the reason for disruption of the gene *glgC* expression

Different cloning strategies were performed to solve the issue about the expression of the gene *glgC*.

3.3.2.1 Modification of the upstream of the gene *glgC* with *glgD* upstream

The upstream 19aa (A') and the overlapping area of 4aa (A'') of the gene *glgD* have been replaced on the modified N-terminal of *glgC* using fusion linker PCR product with different specially designed primer to amplify fragment A of the gene *glgD* (A' + A'' = A), This was done with the forward primer M-D_Bam (P1) and the reverse primer R.M-D_20 (P2) which has a 20bp homology to the linker of the forward primer F.M.C_20up (P3) that was used to delete the first 3 amino acids of the N-terminus including the start codon of *glgC*.

The first step was to amplify the fragment A with primer P1 and P2. The modified *glgC*$_{\_his}$ (fragment B) was amplified with primer P3 and R.His_sac (P4). The PCR products, A and B, were used in fusion PCR reaction to obtain the modified upstream of the *glgC*$_{\_his}$ using the primers P1 and P4. A schematic diagram for the construction of the fusion product *glgC*20up$_{_his}$ carrying the expression vector is shown in Fig. 54. The fusion PCR product *glgC*$_{20}$up$_{_his}$ was purified and cloned with the same cloning strategy used to obtain pH3-M-*D*$_{\_his}$. The resulting plasmid pH3-M-*C*20up$_{_his}$ was proven as described above and the correct construct was transformed and expressed under same condition mentioned earlier. The two advantages of this modifying strategy were that the stop codon of the overlap area in the N-terminus of *glgC* would stop the reading frames of *lacZ* (Fig. 54) and the utilization of the upstream of the *glgD* gene for successful expression of *glgC*.

The supernatant and crude extract fractions of the homologous expression protein GlgC in *L. lactis* MG1363 were analyzed on 12%SDS-PAGE gel. Surprisingly, the protein GlgC$_{20}$up was successfully overproduced under the previously mentioned modification, but unfortunately has been detected in the periplasmic space forming inclusion bodies (Fig. 55).

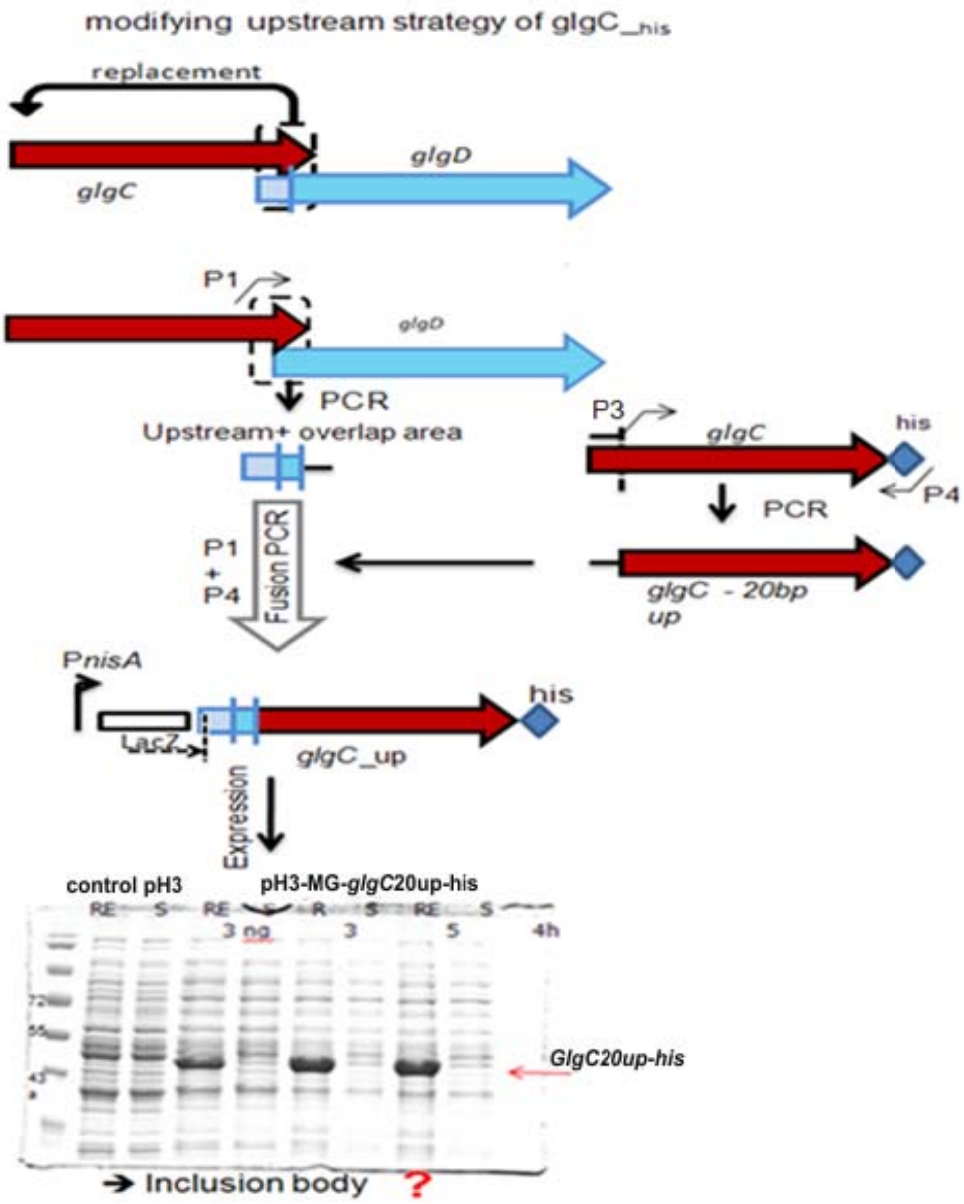


Fig. 55 A: Schematic showing modifying strategy of the gene *glgC* with the overlap and upstream area of the *glgD* using Fusion PCR to replacement these area; B: the expression fraction of the construct pH3-M-C_{20}up_his and as a control empty plasmid pH3 were analyzed on the 12%SDS-gel; RE-crude extract, S-supernatant fraction induced with different final concentration of nisin 1, 3 and 5 ng/ml when the cells reaches the OD_{600}=0.35 for 4 hours at 30°C. The GlgC protein in these experiments was accumulated in the periplasmic space formation insoluble protein as an inclusion bodies form (RE Fraction).

With the obtained knowledge from the first strategy, a new strategy for homologous expression of *glgC* was designed based on the deleting the marker *lacZ* from the Nisin controlled plasmid pH3. Cloning of the gene *glgC*$_{his}$ at the restriction site of *Xba*I and *Sac*II directly downstream of the *nisA* promoter resulted in the expression vector pMil3-MG-*glgC*$_{his}$. The construct was analyzed by restriction digestion before trasforming and expressing into *L. lactis* MG1363 strains. A scheme for the

construction of the new expression vector is shown in Fig 56.

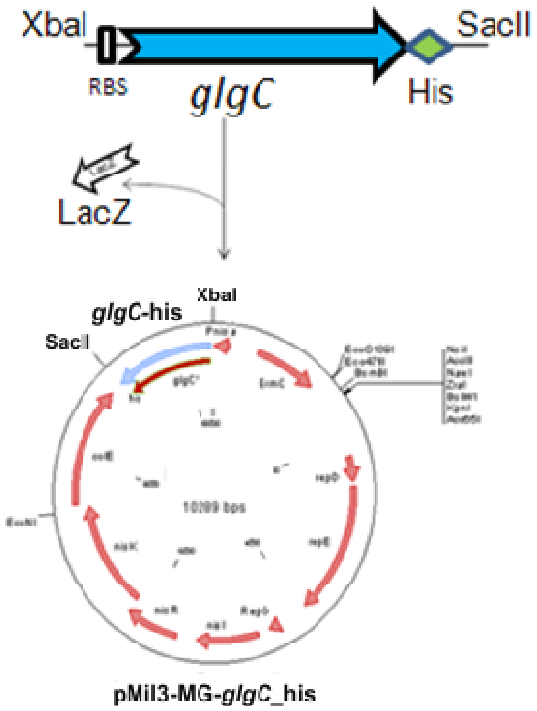


Fig. 56
Schematic of deleting the l*acZ* from the pH3 with *Xba*I and *sac*II enzyme and cloning the gene *glgC*$_{his}$ at the same cut site to obtain at the end new expression plasmid pMil3-MG-*glgC*$_{his}$.

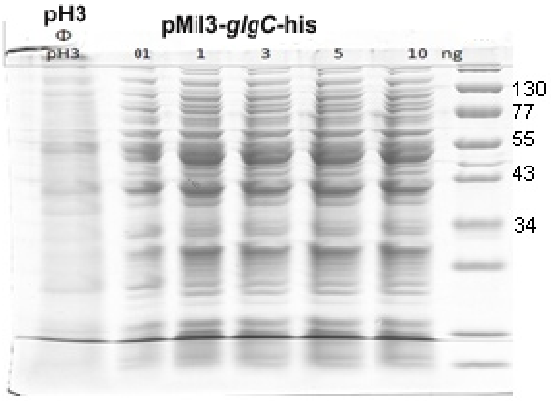


Fig. 57
SDS-PAGE analysis of the homologous expression plasmid pMil3-MG-*glgC*$_{his}$ in *L. lactic* MG1363 at the growth point different amount of nisin was added 0.1, 1, 3, 5 and 10 ng/ml to the culture. The protein bands compared with negative control empty pH3 expressed under same condition and induced with
3 ng nisin/ml

The result of the homologous expression of the construct vector pMil-MG-*glgC*$_{his}$ was analyzed on 12%SDSD-PAGE as described previously and expression of the empty plasmid pH3 under same condition was used as control. As shown in the Fig. 57, no difference between the band intensity was detected for either the construct or the control.

Furthermore, the fragment *glgC*-$_{his}$ including the transcription terminator from construct pH3-MG-*glgC*$_{his}$ was obtained with preparative digestion with the enzymes *Bam*HI and *Xho*I. The agarose purified fragment was cloned into the expression plasmid pMSP3535 that was previously proposed as a version of the nisin plasmid missing a transcriptional

terminator which probably led to the disruption of the expression (Kim and Mill, 2007). The cloning was done at the same enzyme sites to obtain pMK35-MG-*glgC*_his vector construct which was transformed into *E. coli* XL10. The purified construct was analysed with different restriction enzymes before transforming into *L. lactis* MG1363 by electroporation. The correct transformants were selected, grown on erythromycin containing MRS medium and induced by activation of the *nisA* promoter with nisin. Extractions were prepared as described previously and the presence of the target protein were analysed by 12 % SDS-PAGE.

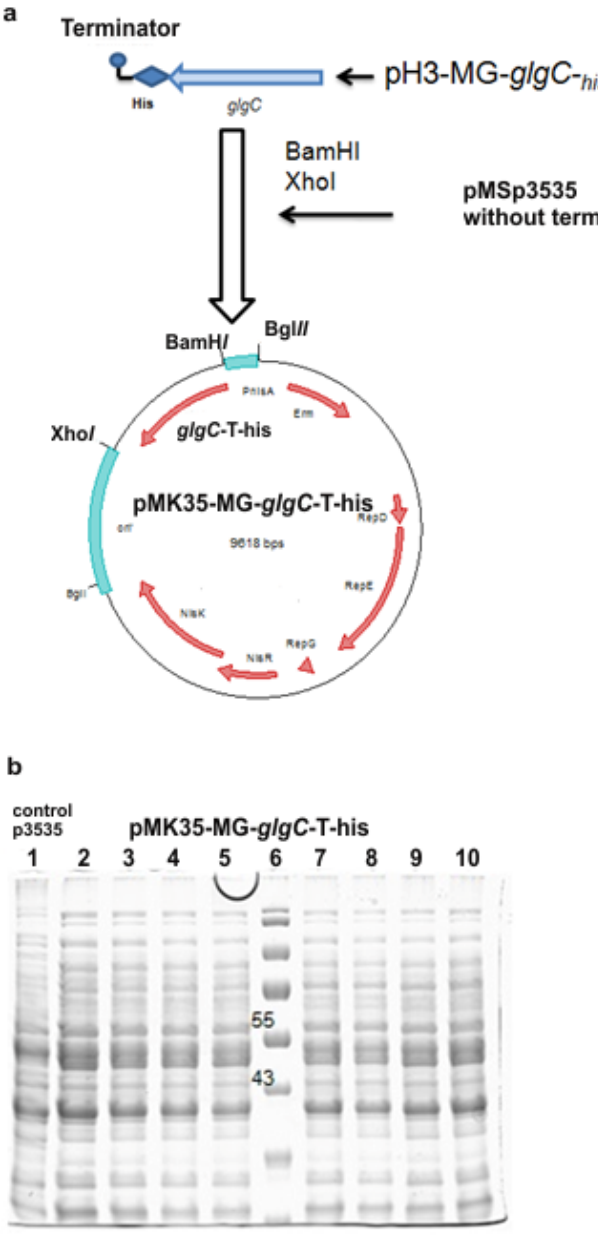


Fig.58. Schematic cloning strategy of the construct pMK35-MG-*glgC*_his from the plasmid pMSP3535, in this cloning the transcription terminator from the expression plasmid pH3 was included in the insert fragment *glgC*_his. The *glgC*_his plus terminator was double digested with *Bam*HI and *Xho*I, purified with 0.8% agarose gel and ligated with purified opened plasmid pMSP3535 at the same cut site of the enzyme, the resulting construct was isolated from *E. coli* XL10 and restriction analyzed before transformed into *L. lactis* MG1363. The empty pMSP3535 plasmid was used as a control.
B. SDS-PAGE analysis of the pMK35-M-*C*_T-his vector under nisin control.1; empty plasmid pH3 as a control.6: protein Marker, 2-10 induced construct with different nisin concentration for 4 h.

In this attempt, no increase in the GlgC protein production was detected. (Fig. 58)
These results exclude our assumption that *lacZ* play a role in the disruption of the gene at the transcriptional or translational level. The previous results obtained were not

sufficient to understand the reason for unsuccessful *glgC* expression.

The +100 bp upstream area of the *glgC* gene seem to be an interesting region which might contain some regulatory element controlling the *glgC* expression.

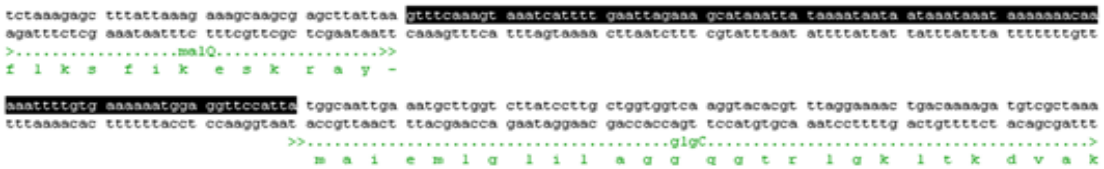


Fig.58c. Schematic showing the upstream area of the *glgC* in Lactococcus lactis strain

To analyze the possibility of the presence of any regulatory element in the +100bp upstream region of *glgC,* the whole region was amplified along with the gene *glgC* using the forward primer F.M.C100up (P1) carrying linker for *Bam*HI enzyme and the reverse primer SK11.C.R (P2) to obtain the PCR product MG-glgC_100 up. This was sub-cloned into the pET101 plasmid in order to obtain the polyhistidine linker at the C-terminus for easier detection and purification. Subsequently, the target gene with 6xHis-Tag was amplified in correct orientation using primer P1 and R.His.Sac (P4) as reverse primer introducing the enzyme site of *Sac*II. The cloning into the nisin plasmid pMSP3535H3 (pH3) was carried out at the same enzyme sites overlapped on the PCR insert (*Bam*HI and *Sac*II respectively). The resulting expression construct was transformed into *E. coli* XL10 and analyzed with restriction enzymes and DNA sequencing for mutation free construct. The correct construct was transformed into *L. lactis* sp.*cremoris* MG1363 by electroporation at (1.5 KV, 25 µF, 300Ω and constant time 4.5-5 ms). The cloning was designed to be sure that the frame reading of the *lacZ* stopped before the transcription start of *glgC* leading to undisrupted gene expression. The resulting expression vector pH3-MG-*glgC*100_his has been expressed under nisin control with different amounts of the inducer nisin 0.1, 3 and 6 ng/ml for 4 hours. The crude extracts and cell supernatant fractions were analyzed on 12% SDS-PAGE gel. In this attempt too, no increase in the expected protein size band has been detected compared to the negative control carrying empty plasmid expressed under same condition (Fig. 59).

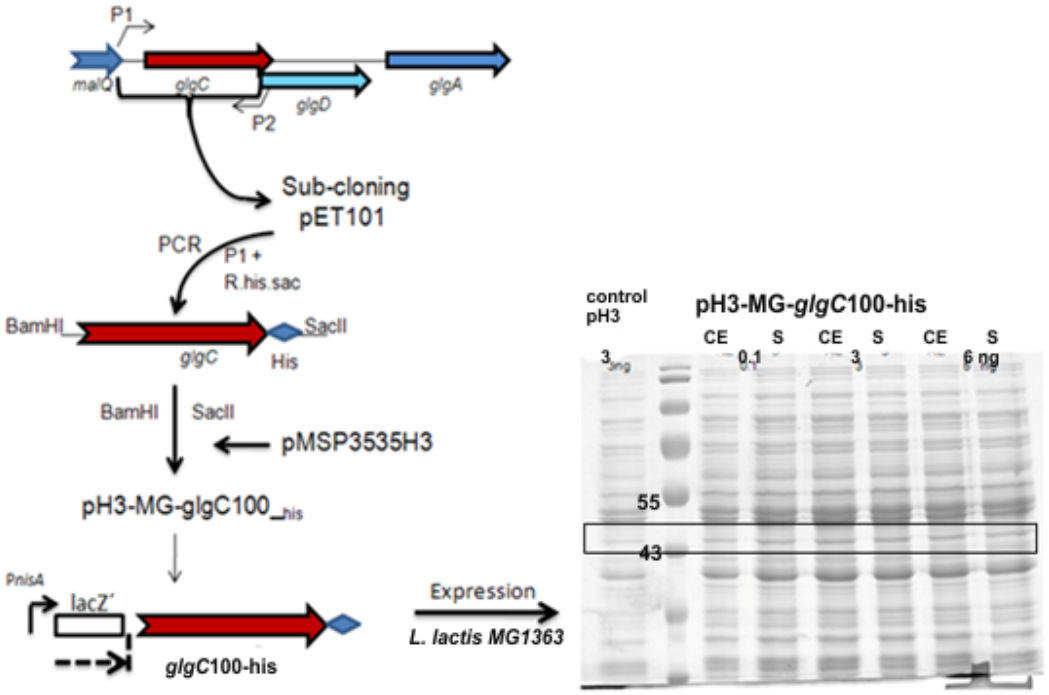


Fig. 59. Schematic showing the expression and cloning strategy of *glgC* and +100 upstream regions into pMSP3535H3. The SDS-PAGE shows the analysis of the pH3-MG-*glgC*_100-his construct under nisin control with different nisin concentration 0.1, 3 and 6 ng/ml for 4 hours. RE: crude extract and S: supernatant.

The messenger RNA (mRNA) often has an important role in controlling the protein synthesis. It was previously proposed that in addition to the transcription and/or translation efficiency, the plasmid copy number and stability of its mRNA can contribute to the control of the gene expression level. In order to analyze the presence and stability of the mRNA, and if that could be the reason for the unsuccessful expression of the gene *glgC* despite different previous attempts to express a reverse transcription PCR experiment was designed for all constructs which were constructed to express the gene *glgC* (pH3-MG-*glgCD*/*D*/*C*_his, pH3-MG-*glgC*20up-$_{his}$ and pMil3-MG-*glgC*_$_{his}$).The RNA analysis of homologous expression of the gene yielded PCR bands of the expected size (Fig. 60), however the experiments design(design of the primers) does not allow to distinguish between the chromosomally encoded and recombinants plasmid-encoded glgC and/or glgD mRNA

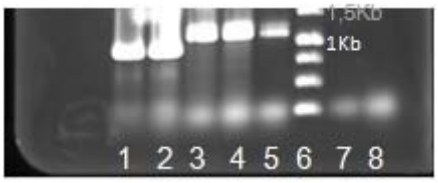


Fig. 60 reverse transcription mRNA -PCR reaction load on 0.8% agarose gel agreement with to expecting size, cells *L.lactis* carrying the following constructs line1: pH3-MG-*glgD*, line2: pH3-MG-*CD*, lane3: pH3-MG-*C*, lane4: pH3-M-*C*20up,lane 5: pMil3-MG-*C*, lane6: DNA Ladder, lane7: control isolated RNA+ Primer (for detection any DNA contamination),lane 8: control without transcriptase enzyme.

Discussion

The glycogen synthesis pathway is already known in various microorganisms (Preiss and Romo, 1994: Ballicora *et al*., 2003). The Glucose-1-phosphate is the actual starting substrate for glycogen synthesis, which upon reacting with the ADP-Glc PPase forms ADP-glucose. The latter serves as the substrate for glycogen synthase (*glgA*), which catalyse the formation of a linear α-glucan or maltodextrins. These get alpha (α-1, 6)–glycosidic linkage with the branching enzyme *glgB* upon repeated cycles to form the ultimate glycogen.

The genomes of the lactic acid bacteria strains *Lactococcus lactis* subsp. *cremoris* IL1403, SK11 and MG1363 have already been completely sequenced. The genes encoding glycogen biosynthesis proteins have been shown to be clustered in a region which includes the four ORFs *glgCDAP.* However, the genes *glgB* and *glgX* encoding the branching enzyme and the debranching enzyme, respectively, were not found within this operon of glycogen synthesis (Fig. 61).

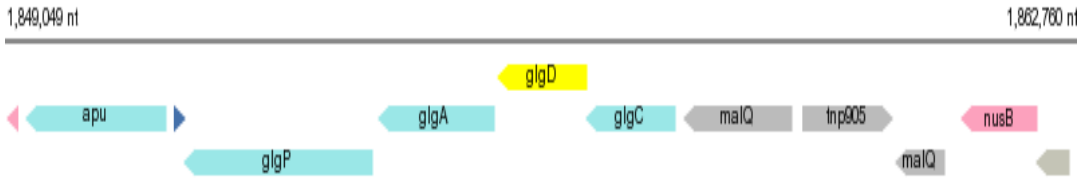


Fig.61. The schematic glycogen genes cluster in *lactococcus lactis* subsp. *cremoris* MG1363

On the other hand, the genes required for glycogen biosynthesis in *Lactobacillus plantarum* WCFS1 have been shown to be clustered in one single region within the operon *glgBCDAP* wherein it was demonstrated that the *glgB* gene encodes branching enzyme (Fig. 62).

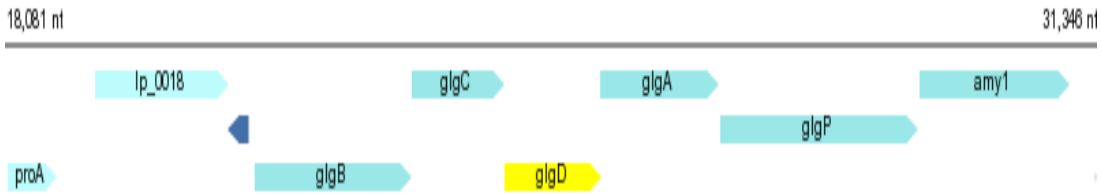


Fig.62. The schematic glycogen genes cluster *in Lactobacillus plantarum* WCFS1

The enzymatic activities of encoded proteins for glycogen biosynthesis have not been analyzed in lactic acid bacteria as of the time of this study.

Discussion

Database analyses and alignment studies with *L. lactis* and *Lb. plantarum* proteins encoded by *glgC* and *glgD* revealed varying levels of similarity to the functionally well-characterized ADP-Glc PPase enzyme. The product of the gene *glgC* shares between 24-36 % identity and 35-54 % similarity with the corresponding gene products from Gram-negative bacteria (e.g. *E. coli glgC)* and Gram-positives *B. subtilis* (50-23 % identity to *glgC-glgD,*respectively) (Table 11). The same genes *glgC* and *glgD* have less similarity to plant ADP-Glc PPase enzyme (about 17-22% identity and 25-30% similarty to the *Arabidopsis thaliana* counterpart). The *L. lactis* subsp. *cremoris* MG1363 and *Lb. plantarum* WCFS1 gene *glgC* (1143, 1140 bp respectively) encodes 380 amino acid protein (42198.9 Da, pI 4.72-5.08).The *L. lactis* subdp.*cremoris* MG1363 and *Lb. plantarum* WCFS1 *glgD* genes (1140 bp, 1173 bp respectively) encode 379 and 390 residue and proteins (42734.2 Da, 43141.8 Da, respectively; pI values 5.47 and 9.13 respectively with limited identity (24-27%) and up to 40-50% similarity to GlgC.

Tab.11. Comparative analyses of the amino acid sequences show the identity score between GlgC and GlgD from different sources *Lb. plantarum* WCFS1, *L .lactis* subsp. *cremoris* MG1363, *E. coli* and *B. subtilis* (B. S).

	Sequence A Name	Size (aa)		Sequence B Name	Size (aa)	identity score
1	WCFS-glgC	379	2	WCFS1-glgD	390	22
1	WCFS-glgC	379	3	E.coli-glgC	431	38
1	WCFS-glgC	379	4	MG-glgC	380	64
1	WCFS-glgC	379	5	MG-glgD	379	22
1	WCFS-glgC	379	6	B.S-C	380	50
1	WCFS-glgC	379	7	B.S_D	343	23
2	WCFS1-glgD	390	3	E.coli-glgC	431	18
2	WCFS1-glgD	390	4	MG-glgC	380	22
2	WCFS1-glgD	390	5	MG-glgD	379	32
2	WCFS1-glgD	390	6	B.S-C	380	20
2	WCFS1-glgD	390	7	B.S_D	343	32
3	E.coli-glgC	431	4	MG-glgC	380	39
3	E.coli-glgC	431	5	MG-glgD	379	16
3	E.coli-glgC	431	6	B.S-C	380	40
3	E.coli-glgC	431	7	B.S_D	343	20
4	MG-glgC	380	5	MG-glgD	379	23
4	MG-glgC	380	6	B.S-C	380	48
4	MG-glgC	380	7	B.S	343	25
5	MG-glgD	379	6	B.S-C	380	20
5	MG-glgD	379	7	B.S_D	343	27
6	B.S-C	380	7	B.S_D	343	26

Discussion

The amino acids Tyr-114 and Lys-195 have been identified to be important substrate binding sites of the ADP-Glc-PPase enzyme from *E. coli*, and it has been found the Lys-39 to be important for allosteric effectors binding (Preiss, J. 1993). A sequence motif, PAV (Pro-52, Ala-53, and Val-54), which is present in every ADP-Glc-PPase known to date, is also important for activator binding (Greene *et al*., 1996). Most of the important PAV motifs and the substrate-binding Lys-195 residue are conserved only in the GlgC sequence. The important GlgC amino acid residues mentioned above are not conserved in GlgD of both *Lactococcus* and *Lactobacillus* strains (Fig.63 and 64).

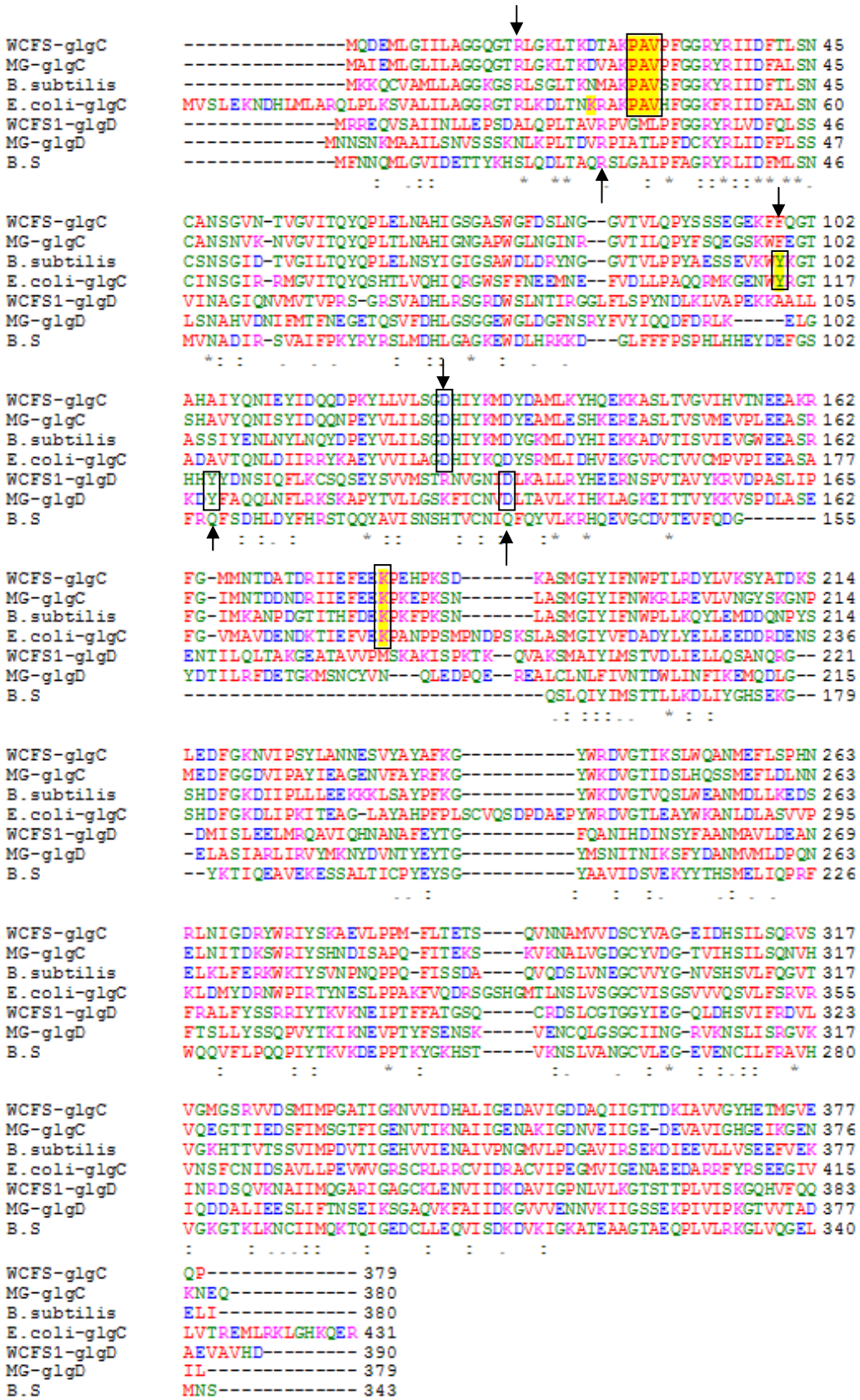


Fig. 63. Amino acid sequence alignment of GlgC (*L. lactis* MG1363 and *Lb. plantarum* WCFS1) with that of ADP-Glc-PPase from *E. coli* and *B. subtilis.* Black boxes indicate identical residues and motif PAV amino acids which are thought to be involved in substrate or activator binding.

Sequence A	Name	Size (aa)	Sequence B	Name	Size (aa)	identity score
1	WCFS1-glgD	390	2	MG-glgD	379	32
1	WCFS1-glgD	390	3	B.S	343	32
2	MG-glgD	379	3	B.S	343	27

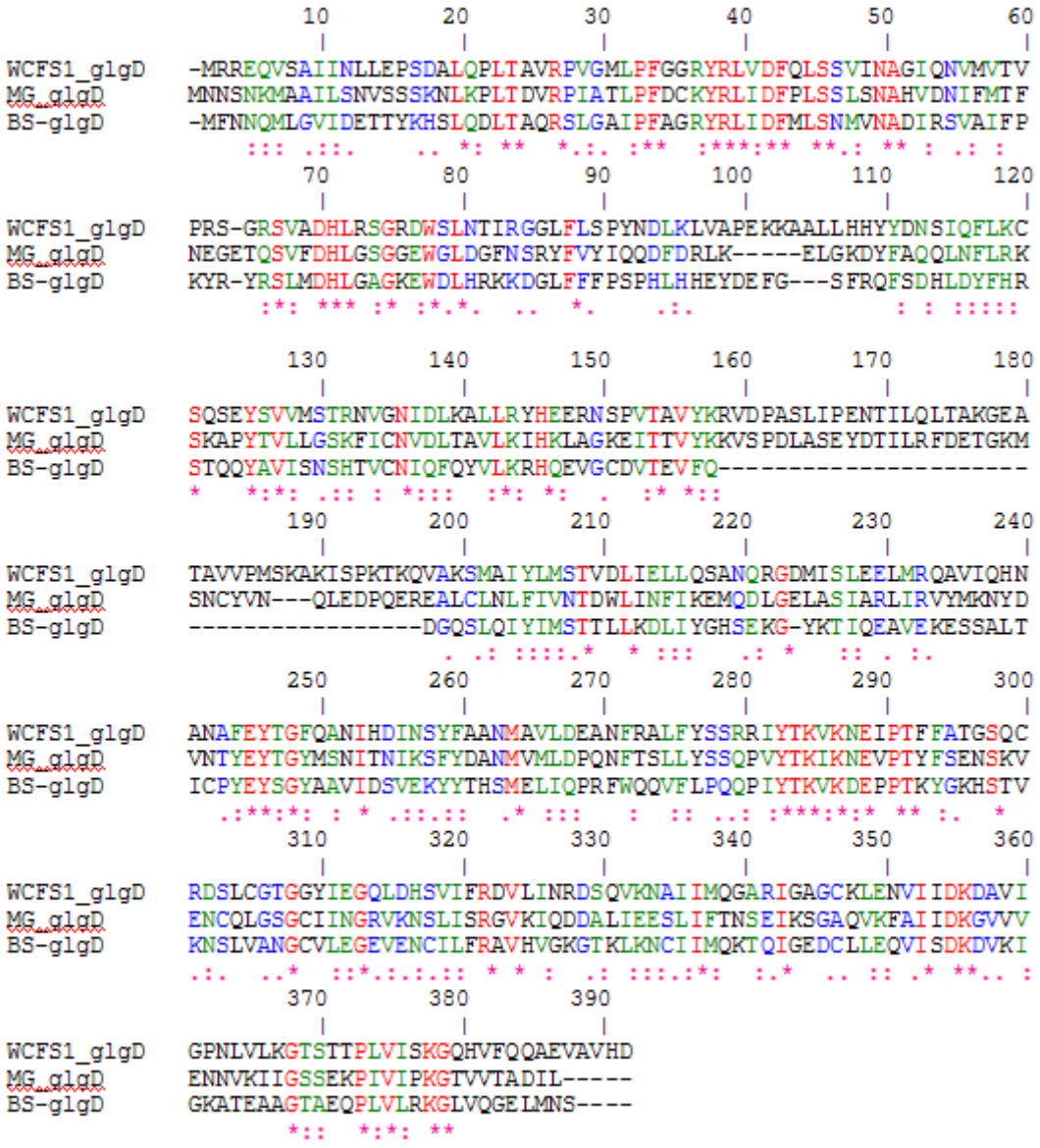


Fig. 64. Amino acid sequence alignment of amino acid sequences of GlgD (MG1363 and WCFS1) with the corresponding sequence from *B.subtilis* (*BS*)

Database analyses and alignment studies with the proteins encoded by *glgC* and *glgD* in both the strains revealed a low level of amino acid sequences similarity (Table 8 and 9) of the proteins with ADP-Glc-PPase. The study by Ballicora *et al*. (2003) had shown the amino acid regions involved in the catalysis and regulation in *E. coli*

ADP-Glc-PPase. The residues Asp142 and Arg32 were proposed to be involved in the catalytic role in the *E. coli* enzyme which corresponds to Asp133 and Arg26, respectively, in the GlgD proteins of both the LAB strains. Lys195 known to have a role in binding of glucose-1-phosphate corresponds to the Lys194 in GlgD protein of *Lb. plantarum.* Tyr114 in the *E. coli* enzyme is involved in for ATP substrate binding and corresponds to the residues Lys105 and 108 in *Lactococcus* and *Lactobacillus*, respectively. The presence of above mentioned residues might be intruiging with respect to the studies on the active site residues in GlgD.

The enzymatic activities of the glg proteins until now have not been analyzed in LAB. Especially, the function of *glgC*-homologous *glgD* gene is unclear. Since *glgD* is found only in some Gram-positive bacteria, it may be important for glycogen biosynthesis in some species of LAB that are able to accumulate glycogen. These species are recognized as bacteria beneficial to human health with probiotic characteristics.

It has been reported that glycogen or granulose serves as an endogenous source of carbon and energy for the bacteria. These bacteria which accumulate glycogen have an advantage to grow under starvation conditions compared to those which are unable to synthesize this storage polysaccharide e.g. *E. coli, S. enterica* and *C. glutamicum* (Alisdair *et. al*., 2005; Seibold *et al.,* 2007). However, in case of the oral bacterium *Streptococcus mutans*, the intracellular polysaccharide (IPS) plays a role in the development of dental caries by enhancing the bacterial survival up to 15 days under starvation phase compared to the glycogen deficient strain (Monica *et al*., 2009). Sporulating microorganisms (e.g. *Clostridium*, *Bacillus*) are able to accumulate glycogen just before the spore formation. This suggests that glycogen plays a role in the survival of the bacteria during starvation, providing energy and carbon for the bacteria and for the spore formation. A significant role for storage glycogen in tolerance to salt or oxidative stress has been suggested for *Synechococcus elongatus* based on studies with *glgC* deletion mutants (Suzuki *et al*., 2010).

Some of the LAB species have the glycogen biosynthesis loci, especially those isolated from the mammalian intestinal system or recognized as probiotics. All previous researches on the survival of the probiotic bacteria indicate the ability of these strains to utilize the oligosaccharide that feed them and encourage their growth in the colon region (Macfarlane *et al*., 1999; Handan *et al*., 2000; Saminathan *et al*., 2011). It is hypothesized here that glycogen may help the bacteria to survive in the intestinal system. For these reasons and according to our results it might be so that the *glg* genes are even essential for the growth of theses strains.

Discussion

4.1 Heterologous expression of the *glg* genes from LAB in the Gram-negative bacterium *E.coli*:

The first strategy was to introduce the genes of interest in *E. coli* by using the pET expression system in order to achieve high level production for the subsequent characterization and functional analysis of the target proteins. In this step, the overexpressed gene products were aggregated in the cells as inclusion bodies. Many attempts including changing the parameters of temperature, stress, growth and coexpression of molecular chaperones as well as fusion expression using MBP (under normal growth conditions) were applied to lower the risk of accumulation of inclusion bodies and increase the soluble form of the protein without any success as shown previously in this study.

The Gram-negative bacterium *E. coli* is the most commonly used organism for heterologous gene expression. Disadvantages of high level overexpression are that the recombinant protein is accumulated in huge quantity either in the cytoplasm or the periplasmic space in the inactive form as inclusion bodies. Many factors such as a strong promoter on a multiple-copy plasmid as well as rare codon usage can play a role in causing problems for high level expression and may be the underlying reasons for inclusion body formation (Schein and Noteborn 1988). To avoid the folding problems often encountered upon microbial overexpression, a speical host strains with similar codon usage optimal for expression in *E. coli* BL21 plysS, i. e. codon plus and Rosetta strains were used albeit without any success.

In addition, the fusion expression was applied in the current study as an attempt to obtain soluble target proteins which yielded same promising results. Under standard growth conditions the fusion MBP expression proteins were accumulated in the form of inclusion bodies, whereas during expression under mixed-stress conditions an increase in the soluble protein fraction up to 50% was successfully done.

4.2 GlgC-GlgD interaction

The purification and experimental analysis of the fusion expression of both the genes *glgC* and *glgD* from *Lb. plantarum* WCFS1 in the current study (section 3.1.6) have shown that the GlgC and GlgD proteins clearly interact with each other. Upon amylose affinity chromatography of the fusion of MBP with the target protein, both bands were surprisingly obtained upon SDS-PAGE analyses, demonstrating that GlgC and GlgD

had been co-purifed. This was confirmed on the Western blot and MS analysis of the SDS-PAGE protein spots, which identified the second band as the His-tagged GlgD protein. To exclude the probability that the GlgD proteinitself had some kind of affinity to the amylose resin, the soluble expression fraction was purified through a nickel-chelating resin to purify the His-tagged protein GlgD_his. Again a co-purified load appeared, this time the fusion protein MBP-GlgC, the result again suggesting a protein: protein interaction. Such strong affinity was again proven by purifying the complex soluble fraction under denaturing condition whereas with a noticeable decrease in intensity of the bands from 3:1 to 1:1 of the proteins. The establishment of the interaction was carried out by proving the affinity between the fusion protein-GlgC expressed from a new construct (pMAl3-W-C) after deleting the *glgD* and the native protein GlgD. Previously, it was suggested in *B. stearothermophilus* that the ADP-Glc-PPase encoded by two genes *glgC* and *glgD* has an $\alpha_2\beta_2$ heterotetramer type structure (GlgCD complex) (Takata *et al.,* 1997). Moreover, in *E. coli* it was proposed that the enzyme, encoded by only *glgC*, consists of two strongly interacting domains (N-/C-terminus) that have to be expressed together to obtain a fully functional enzyme and also could not be separated by chromatographic procedures (Clarisa *et al*., 2004). A corresponding result was obtained in this work in which an interaction between the GlgC and GlgD proteins was evident and there were difficulties to separate both proteins by chromatography procedures. Thus, in our case, this indicates that both proteins are subunits of the enzyme forming the fully active protein and might suggest a comparable model of α/β heteroligomer type structure. To our knowledge, this is the first study to prove that the GlgC: GlgD proteins have an interaction in lactic acid bacteria, perhabs similar to what was suggested for *B. stearothermophilus* (Takara *et al.*, 1997).

The missing activity of the purified GlgCD complex proteins *in vitro* might suggest that under normal growth conditions some factor(s) stimulate the assembly *in vivo*, which might have been lost during the purification steps but which play an important role in retaining the biological activity of the purified target protein. To clarify these possibilities, further activity analyses of the purified protein complex under different conditions including the supplementation of crude extract from the wild type strain to provide any missing factors that may be needed for enzyme activation are required. Also other possibilities for insufficient activity might be that the protein was not completely correctly folded.

4.3 Heterologous expression in Gram-positive bacteria

Alternative hosts like *Bacillus* and *Corynebacterium* strains have become more and more attractive for heterologous protein production. One reason is by using different hosts that differ in their codon usage preferences and other gene expression features one can attempt to reduce this problem of expression level of the proteins. With this strategy the expression plasmid carrying the genes of interest pWLQ2 was introduced in *C. glutamicum* and pHCM05-WCFS1-*glgC*_his in *B. subtilis* but without any success at the expression level which remains unresolved but may be accounted for example by the instability of the mRNA transcript or a low frequency of the mRNA translation. In the case of introducing the coupled *Lb. plantarum* WCFS1 *glgC* and *glgD* genes carried on plasmid pHMC05 in *B. subtilis*, a large fragment of the target gene insert was deleted from the plasmid construct. Database analyses and alignment studies show that the identity between the genes in both the strains was between 50-23% for *glgC* and *glgD*, respectively. This gene similarity may explain the fragment missing from the insert due to recombination. Alternatively, the gene insert may have been digested via endonuclease restriction and/or integration into the host chromosomal DNA by an unknown mechanism.

4.4 Homologous expression of the genes

The NICE control expression system offers certain advantages with respect to the expression of the heterologous and homologous genes in soluble form. For this strategy, the vector pMSP3535 was used for the homologous expression of the genes *glgC* and *glgD* from *L. lactis* MG1363 and for the heterologous expression open reading frames namely ORF 1 through 3 from *Lb. sanfranciscensis*. It turned out that the genes *glgC* and ORF1-2 did not successfully overexpress, whereas an interesting increase in the intensity of protein bands was detected for the expression of the genes *glgD* and *ORF*1 (*Amy*). The explanation may lie in the presence of the native transcription terminator that coincidentally amplified the 50 bp downstream *glgD* gene region which has been shown to have a high probability of the presence of native transcription-terminator. This corresponds with a speculation previously brought forward by Kim and Mills (2007) to expain the non-expression or very low expression level where they assumed that this version of pMSP3535 missed a transcriptional

terminator which probably leads to the disruption of the expression of *nisK* and *nisR*, the essential genes of the NICE expression system (Fig. 65).

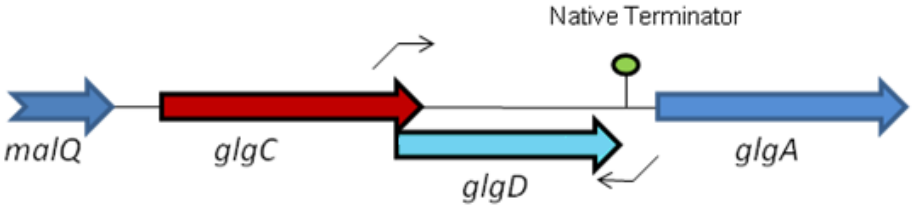


Fig.65. Schematic explains the reason of successfully expression of protein GlgD using the bioinformatics analysis which confirm previously proposed by Kim and Mills (2007).

The improved version pMSP3535H3 (Gian *et al*., 2009) was used to circumvent the problems of the old version and to introduce the modified C-terminal of genes *glgC* and *glgD* from *L. lactis* MG1363 strain separately and simultaneously with histidine-tag in *L. lactis* MG1363. In the study reported that, the gene *glgD* product was successfully over-expressed and purified. The purified GlgD protein showed low activity with the substrate glucose-1-P substrate to produce ADP-glucose. This slight activity may be accounted for by the partial restoration or substitution of the function of the ADP-Glc-PPase, which is encoded usually by the *glgC* gene.

In a previous work, it has been reported that the *glgD* gene is found only in *Bacillus glg* operon residing in the *glgBCDAP* cluster and may be important in glycogen biosynthesis (Kiel *et al*., 1994). In *B. stearothermophilus*, the ADP-Glc-PPase is a heterotetrameric enzyme consisting of the GlgC and GlgD proteins, which are homologous to each other (Takata *et.al*., 1997). Although the GlgC protein has a catalytic activity without GlgD, the latter did not show any significant activity whereas the GlgC activity was lower than that of the complex GlgCD enzyme in heterotetrameric structure (Takata *et al*., 1997). The function of *glgD*, therefore, is unclear but the gene product GlgD was suggested to be a regulator for the catalytic GlgC (Takata *et al*., 1997).

Moreover, strong evidences have shown that the *glgC*-mutant of *E. coli* and Gram-positive bacterium *Streptomyces* accumulates glycogen (Martin *et al.,* 1997; Gustavo *et al*., 2007). On the contrary, the deletion mutants Δ*glgCAP* of *E. coli and S. enterica* were totally devoid of glycogen but accumulate ADP-glucose, which confirmed the involvement of genes other than *glgC* in glycogen biosynthesis of the *Enterobacteria ceae* (Maria *et al*., 2007).

In higher plants and unicellular algae, starch biosynthesis is controlled by heterotetrameric ADP-Glc-PPase which comprises two distinct homologous subunits, small (S) and large (L) (S_2L_2). It was found that the small subunit homotetramer in

Discussion

Solanum tuberersum (StuS), *Arabidopsis thaliana* (APS1) and *Hordeum vulagre* have a catalytic function with defective regulatory properties in some cases (Ballicora *et al*., 2003, 2005; Doan *et al*., 1999) whereas the regulatory subunit L from potato tuber (StuL) could have deficient catalytic function of the StuS (Ballicora *et al*., 2005). The Arabidopsis APL1 and APL2 isoforms exhibit both the regulatory and catalytic functions with distinctive sensitivities to the allosteric activator 3-phosphoglycerate whereas the APL3 and APL4 behave like StuL (Crevillen *et al*., 2003; Ventriglia *et al*., 2008). The maize (*Zea mays*) endosperm L subunit has been postulated to have a role in catalysis due to the effects on apparent affinity of the substrates (Cross *et al*., 2004). In unicellular algae, *Ostreococcus tauri* the L subunit has been suggested to contribute a role in the catalytic effect based on the high affinity for 3-phosphoglycerate and substrates in presence of an S subunit mutant (Misty *et al*., 2009).

In our current study, a low enzymatic activity of the GlgD protein was observed which lead to the production of ADP-glucose in small amounts from Glc-1-P in presence of ATP. This ADP-glucose is the basic substrate in the bacterial glycogen biosynthetic process and is produced by the enzymatic activity of GlgC and its homologues in other bacteria. Thus, the ability of GlgD to catalyze this conversion in the LAB indicates its possibility that it could take over the catalytic role in absence of GlgC for some unknown reason. One of the plausible explanations for the observation could be that the GlgD protein acts as the subunit of a protein complex with GlgC and thus, when alone, renders some minimal activity as observed in our study. The other possibility could be that the GlgC and GlgD being quite homologous possess similar function. As essential functions are generally protected by repetition so that the loss of one protein can be compensated for by the activity of another (Helena *et al*., 2006) it might be that GlgD takes up the function of GlgC, albeit, in a reduced manner.

Highly conserved proteins are often required for basic cellular function, stability or reproduction. Conservation of protein is indicated by the presence of identical amino acid residues at the analogous sites of proteins as well as by the presence of functionally equivalent residues. Thus, removal of only one of the repeated genes could theoretically preserve the function on the assumption that the other conserved sequence is sufficient enough to carry out the essential functional process of the organism. It might be that these new catalytic and/or regulatory roles of the GlgD protein could be the result of the divergence during the course of evolution from a common ancestor enzyme.

On the other hand, the interaction between the proteins GlgC and GlgD has been shown in the current study. Therefore it could be so that during the homologous expression of *glgD*, the overproduced protein has interacted with the native GlgC protein from the host cell and the complex thus formed passed through the hisitidine purification column. This might be another explanation for our observation of low-level ADP-Glc-PPase activity when assayed for the activity of the protein GlgD.

The unsuccessful expression of the gene *glgC* leads us to follow a different strategy to obtain the protein. However, modification of the N-terminus of the gene *glgC* in our attempts to overproduce has led to the expression of the target GlgC protein but in an inactive form as inclusion bodies. As many element can control the stability of transcripts including RNase, regulatory small RNAs and RNA binding-protein which bind to 5-untransalted leader (5´UTR) of the target transcripts and inhibit and/or regulate the expression at the translational level, the region for RNA-binding motifs and their target sequences are also essential for tight protein-RNA association and proper gene regulation. Thus, any change in the sequence will lead to the inability to bind RNA. Hence, in our attempt with engineered *glgC*, it might be so that the binding site has been deleted upon modifying the N-terminus of the *glgC* with the overlapping region. This finds support from the observation that the 5′-untranslated leader of *glgCAP* contains two previously characterized target sites in the global regulatory protein CsrA which has been reported as the repressor by Jeffrey *et al.* in 2009. On the other hand, it might also find an explanation from the observation of Clarisa *et al*. in 2004 in *E. coli* where a strong interaction between the N- and C-terminal domains is required to express the soluble protein and to obtain the fully functional enzyme

Moreover, it was also found in unicellular algae *Ostreococcus tauri* that the presence of both the subunits S and L of ADP-Glc-PPase is necessary for the stability of the heterotetramer (Giroux *et al*., 1994; Wang *et al*., 1998). On a similar note, the observation that the L subunit needs the S subunit for soluble expression (Misty *et al*., 2009) might support our assumption of an important element required for the stability and/or solubility for the expression of the *glgC* and *glgD* genes at their overlapping region. Such overlap region including the stop codon of *glgC* and start codon of *glgD* suggested a coupled gene transcription.

In an attempt to analyze the problem arising from the experiment on N-terminus modification of GlgC, it was thought to be the stop codon of the overlap region which might play a role to stop the disruption of the *glgC* transcription. However, our observation from the results section 3.3.2.1 excludes this assumption, which led us to

confirm our assumption that the overlap region has some unknown transcription/translation element required for the soluble protein expression. Nevertheless, the formation of permanent protein-protein interaction between GlgC and GlgD complex may be considered a part of the protein folding process. As the properties of this complex interface are unique for each interaction pair of GlgC and GlgD, it could be considered as analogous of the fully active site of the ADP-Glc-PPase enzyme.

Moreover, the activity that appeared in enzyme assay of the purified GlgD protein of *L. lactis* MG1363 in presence of UTP instead of ATP indicate a protein contamination in the elution fraction of protein GlgD during purification process or an affinity of the protein GlgD to different nucleotide triphosphate as a substrates. This in turn indicates the ability of the GlgD protein to synthesize both UDP-glucose and ADP-glucose in absence of the catalytic GlgC activity. However, no activity was detected from the complex GlgCD protein in *B. stearothermophilus* with the UTP in placed of ATP (Takata *et al*., 1997).

Several previous reports have shown that the protein GalU, a nucleotide-sugar pyrophosphorylase, considered to specifically recognizing Glc-1-P and UTP to produce UDP-glucose (UDPG) (Nakae *et al*., 1971), can also produce ADP-glucose from Glc-1-phosphate in presence of ATP (Ritter *et al*., 1996; Zea *et al*., 2002). As mentioned previously and from our observation, we suggest an alternative pathway for the synthesis or increase in the production of the UDP-Glucose which is usually used as the subunit in the Exo-polysaccharide (EPS) biosynthesis and may be used by glycogen synthase (GlgA) to accumulate the glycogen. GlgA might be able to utilize not only the ADP-glucose but also UDP-glucose as a glycosyl donor in the biosynthesis of glycogen as indicated in *B. stearothermophilus*, *Mycobacterium tuberculosis* and some archaeal species which seem to use both of the sugar nucleotides (König *et al*., 1982; Pellerin *et al*., 1987; Tounkang *et al*., 2008). However, the main exception in glycogen biosynthesis was in *Prevotella bryantii* that lack ADP-Glc-PPase and whose GlgA exclusively recognize UDP-Glc as the glycosyl donor (Lou *et al*., 1997). Thus, our suggested alternative pathway in *Lactococcus* might also explain the increase of glycogen accumulation in case of deletion of *glgC* compared with the deletion of *glgD* in *B. stearothermophilus* by Takata *et al*., 1997. The schematic show our suggest alternative pathway shown in Fig.66

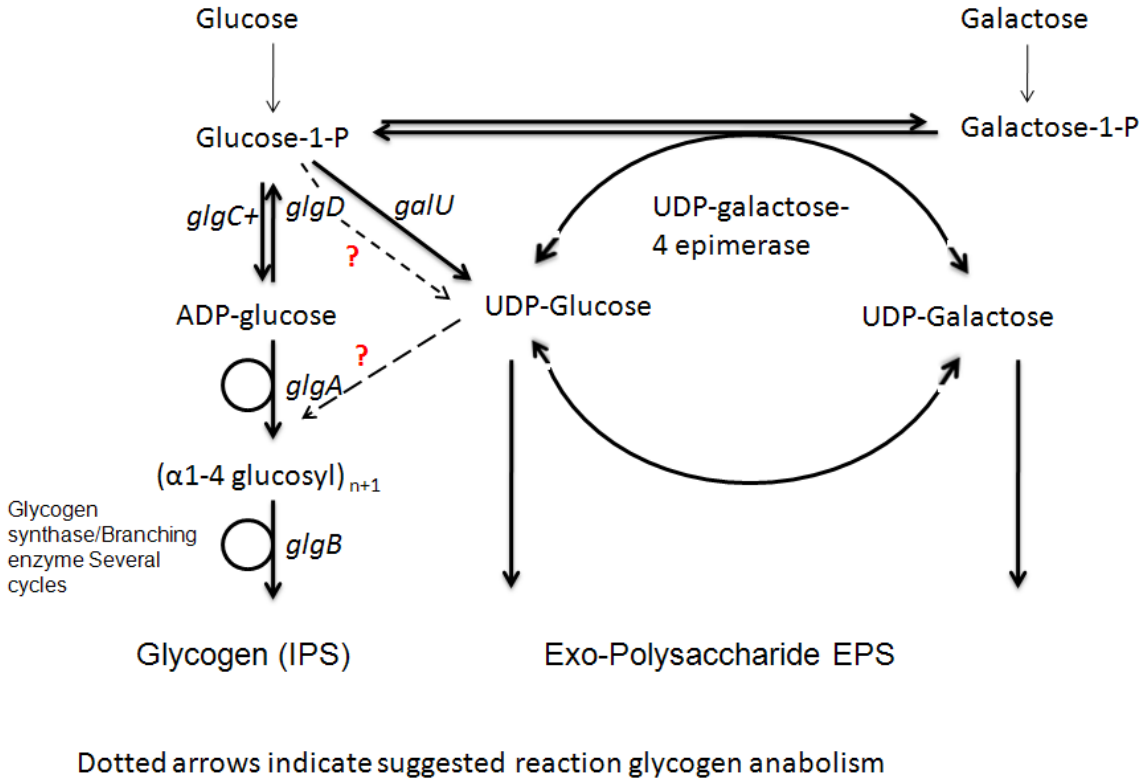


Fig. 66 Schematic diagram for the glycogen pathway and the suggested alternative one

The iodine coloration of the *L. lactis* MG1363 in our attempts with different knocking out vectors to disrupt the gene *glgD and glgC* had shown a phenotype of cream color compared with the light brown in case of attempts for *glgC* mutation by using the *Lacotbacillus* vector pME1. However, there was no success upon using other vectors to knock out the genes. This observation was not in agreement with that shown earlier (Takata *et al*., 1997) in *B. stearothermophilus* where mutations in *glgC* and/or *glgD* resulted in a significant decrease in the yield of glycogen production and suggested that both of these genes are needed for glycogen synthesis. They did not know the reason for the higher level of glycogen in the *glgC* mutant which gave a light brown color compared with those in the *glgD* (cream yellow) mutant cells. This result might indicate to an important function of the GlgD protein in the glycogen biosynthesis of *L. lactis.*

As shown in our results, the unsuccessful knocking out of the genes *glgC* and *glgD* might be accounted for by the diploid chromosomal DNA of the *L. lactis* subsp. *cremoris* MG1363. A knock-out in one chromosome might be compensated for by the intact gene in the other which is possible in case of genes which are essential. Thus, being essential for the growth and survival of the lactic acid bacteria, it indicates

important roles of the storage polysaccharide glycogen. It might give an advantage by enhancing the bacterial stability and survival in gastrointestinal system.

The unsuccessfulness in knocking out these genes could also be attributed to the instability of the vectors used in the current study. For this, a new knockout plasmid based on the sensitive origin is recommended for the disruption of the genes in *L. lactis* strains. However, more experiments are required at this level to obtain the same.

Summary

The polysaccharide glycogen has been reported in many bacteria as well as in eukaryotes. It contains only glucose moieties linked via α-1, 4 linkages and is branched via α-1, 6 linkages. Generally, it is considered to be a storage compound providing both carbon and energy under starvation conditions for the maintenance of cell integrity and the preservation of necessary metabolic processes by protecting some cellular constituents.

In bacteria, regulation of glycogen biosynthesis is achieved by controlling the expression of the *glg* genes. The first step of the synthesis is the formation of ADP-glucose from glucose-1-phosphate by ADP-glucose pyrophosphorylase (ADP-Glc-PPase; ATP: α-D-glucose -1-phosphate adenylyltransferase, EC 2.7.7.27) encoded by *glgC*. This is followed by glycogen synthase (EC 2.4.1.21) and the branching enzyme (EC 2.4.1.18) encoded by the *glgA* and *glgB* genes, respectively. The key regulation step of glycogen biosynthesis in prokaryotic organisms, which has been investigated extensively, is the reaction catalyzed by ADP-Glc-PPase which forms ADP-glucose and pyrophosphate from ATP and D-glucose-1-phosphate.

A comparative analysis of the glycogen biosynthesis gene cluster in the Gram-negative and Gram-positive bacteria shows that some Gram-positive species of *Bacillus, Clostridium* and lactic acid bacteria (LAB) have two genes *glgC* and *glgD* encoding proteins similar to ADP-Glc PPase. It has been reported that GlgC and GlgD comprise the subunits of an $\alpha_2\beta_2$-type heterotetrameric structure. However, the role of the additional *glgD* in the Gram-positives is poorly understood. Only a regulatory role of *glgD* has been reported in *Bacillus stearothermophilus* without any enzymatic activity having been shown for the protein product GlgD. In *Bacillus subtilis* and *Streptomyces coelicolor*, glycogen synthesis has been associated with sporulation and the supply of resources necessary to drive differentiation. Regarding the enzymatic activities of the Glg proteins, especially the function of GlgD which shows amino acid sequence similarity with GlgC is not characterized.

This study deals with the investigation of the *glg* genes in some LAB. Lactic acid bacteria are a group of facultative anaerobic, non-pathogenic, non-colonizing, non-sporulating Gram-positive bacteria which are important for the human health and industrial food processes. We have focused on the detailed functional analysis of both the genes *glgC* and *glgD* residing in the *glgCDAP-B* or *glgBCDAP* operons of *Lactococcus lactis* subsp *cremoris* MG1363 and *Lactobacillus plantarum* WCFS1, respectively. In particular, the investigation of the function of the *glgC*-homologous *glgD* gene in LAB was of interest to understand the role of the storage polysaccharides in

this group of organisms. More information about the role of *glgD* might help to unravel the mechanism of synthesis and/or regulation of glycogen in these bacteria and possibly link intracellular polysaccharide (IPS) formation with probiotic characteristics of certain species of LAB.

Attempts forward the functional characterization of these genes included expression in different engineered *E. coli* strains which was possible for all target proteins as insoluble inclusion bodies. However, various experimental attempts to prevent inclusion bodies and obtain more soluble protein, including expression at low temperature, growth under stress conditions, coexpression with different chaperones and refolding the protein from inclusion bodies, were not successful. Nevertheless, in a fusion expression study of the genes *glgC* and *glgD* from *Lb. plantarum* WCFS1, it was shown under our specific growth conditions that there was an increase in the solubility of the protein fraction up to 50% compared with the standard condition. Experimentally it was proven that the proteins GlgC and GlgD strongly interact with each other. Both proteins appear to be subunits forming the fully active enzyme with a model comprising α and β subunits which might form a heterotetrameric type structure, as was also suggested previously in the Gram-positive bacterium *Bacillus stearothermophilus*.

To our knowledge, this is the first study showing that the GlgC and GlgD proteins interact with each other in lactic acid bacteria. Moreover, in this study, a low ATP-dependent enzymatic activity of the GlgD protein in the absence of GlgC was also observed although the possibility of a small amount of GlgC from the host cell co-purified with the heterologous GlgD can not be completely ruled out. This ability of the GlgD protein to produce ADP-Glc indicates a possible dual catalytic/regulator function of the protein. It is proposed that the ADP-Glc-PPase activity represents a protein complex, encoded by the genes *glgC* and *glgD*. These genes are conserved in these LAB strains (*Lactococcus lactis* subsp *cremoris* MG1363 and *Lactobacillus plantarum* WCFS1). Moreover, the unsuccessful attempts for the expression of GlgC might refer to the essentiality of the presence of GlgD for the stable and soluble expression of *glgC*, perhaps by assisting protein folding. Further investigations are necessary to understand the many regulatory aspects of glycogen metabolism in LAB at both the transcriptional and translation levels. It might also be possible that both these genes are essential for growth of these species as they could not be knocked out with different vectors.

Finally, an important observation in this study was the affinity of the purified GlgD for UTP as a substrate which is indicative of an alternative reaction to produce UDP-

glucose. This may indicate an alternate pathway for glycogen biosynthesis. The observation that the *glgC* and *glgD* genes are apparently essential and that there was exist a new pathway for glycogen biosynthesis indicate that glycogen plays an important role in the survival of these LAB strains.

Zusammenfasung

Das Polysaccharid-Glykogen ist aus vielen Bakterien wie auch Eukaryoten bekannt. Es enthält ausschließlich über α1-4 Bindungen verknüpfte Glucoseeinheiten, die über α1-6-Bindungen verzweigt sind. Generell ist es als ein Speichermolekül anzusehen, das sowohl Kohlenstoff- als auch Energiequelle unter Stressbedingungen darstellt. Es ermöglicht die Aufrechterhaltung der Integrität der Zelle und die Erhaltung der notwendigen Stoffwechselvorgänge und führt zum Schutz einiger Zellbestandteile.

In Bakterien ist die Regulation der Glykogen-Biosynthese durch die Kontrolle der Expression der Gene *glg* möglich. Der erste Schritt der Synthese ist die Bildung von ADP-Glucose aus Glucose-1-Phosphat durch ADP-Glucose-Pyrophosphorylase (ADP-Glc-PPase; ATP: α-d-glucose-1-Phosphat adenylyltransferase, EC 2.7.7.27), kodiert vom Gen *glgC*, gefolgt von den Reaktionen der Glykogen-Synthase (EC 2.4.1.21) und des Verzweigungsenzyms (EC 2.4.1.18), von dem die Genen *glgA* und *glgB* Genen kodiert sind. Der entscheidende Regulierungsschritt der Glykogen-Biosynthese in prokaryotischen Organismen, ist die durch ADP-Glc-PPase katalysierte Reaktion, die zur Bildung von ADP-Glukose und Pyrophosphat aus ATP und D-Glucose-1-Phosphat führt.

Eine vergleichende Analyse des Glykogen-Biosynthese-Genclusters in Gram-negativen und Gram-positiven Bakterien zeigte, dass einige Gram-positive Spezies aus der Gattung *Bacillus* und *Clostridium* und den Milchsäurebakterien zwei Gene (*glgC* und *glgD)* besitzen. Sie kodieren für Proteine, die der ADP-Glc PPase ähnlich sind. Es wurde dokumentiert, dass GlgC und GlgD die Untereinheiten eines $\alpha_2\beta_2$-Typ heterotetrameren Struktur bilden. Allerdings ist die Rolle von *glgD* bei Gram-positiven Bakterien noch unklar. Nur eine regulatorische Rolle des *glgD* in *Bacillus stearothermophilus*, ohne erkennbare enzymatische Aktivität des Protein-Produkts von GlgD ist bisher bekannt. In *Bacillus subtilis* und *Streptomyces coelicolor* hängt die Glykogen-Synthese mit der Sporulation und der Versorgung mit notwendigen Ressourcen zur Differenzierung zusammen. Die enzymatischen Aktivitäten der Glg Proteine, vor allem die Funktion von GlgD, welches Ähnlichkeiten in der Aminosäurensequenz mit GlgC zeigt, sind nicht charakterisiert. Demzufolge war der Schwerpunkt dieser Arbeit auf die Untersuchung der Funktion des glgC-homologen *glgD* Gens in einigen Michsäurebakterien (lactic acid bacteria, LAB) gerichtet. LAB sind eine Gruppe von fakultativ anaeroben, nicht pathogenen, nicht sporenbildenden grampositiven Bakterien mit wichtigen Funktionen für die menschliche Gesundheit und die Lebensmittelindustrie.

Die vorliegende Arbeit ist auf die detaillierte funktionelle Analyse der beiden Gene glgC

und *glgD* konzentriert, welche in den *glgCDAP-B* bzw. *glgBCDAP* Operons von *Lactococcus lactis* subsp. *cremoris* MG1363 und *Lactobacillus* plantarum WCFS1 liegen. Insbesondere war die Untersuchung der Funktion des glgC-homologen *glgD* Gens in LAB von Interesse für das Verständnis der Funktion des Speicher-Polysaccharids in dieser Organismengruppe. Mehr Informationen über die Funktion von *glgD* könnten dazu beitragen, den Mechanismus der Synthese und / oder Regulierung von Glykogen in dieser Bakteriengruppe zu verstehen und möglicherweise intrazelluläre Polysaccharidbildung (IPS) mit probiotischen Eigenschaften bestimmter Arten von LAB zu verknüpfen.

Versuche zur funktionellen Charakterisierung dieser Gene schlossen derer Expression in verschiedenen *E. coli* Stämmen ein, dies gelang für alle Zielproteine in Form von unlöslichen Inclusion-bodies. Es wurden verschiedene Versuche unternommen, um die Bildung von Inclusion-bodies zu verhindern und eine größere Menge an löslichem Protein zu erhalten. Verschiedene Ansätze, wie Expression bei niedrigen Temperaturen, Wachstum unter Stress und Co-Expression mit verschiedenen Chaperonen sowie die Rückfaltung des Proteins aus Inclusion-bodies, waren nicht erfolgreich. Es war jedoch möglich, mittels einer Fusionsexpressionsuntersuchung der Gene *glgC* und *glgD* von *Lb. plantarum* WCFS1zu zeigen, dass es unter speziellen Wachstumsbedingungen eine Zunahme der Löslichkeit der Proteinfraktion um bis zu 50% im Vergleich zu den Standard-Zustand gab. Experimentell wurde nachgewiesen, dass die Proteine GlgC und GlgD stark miteinander interagieren. Beide Proteine scheinen Untereinheiten zu sein, die das voll aktive Enzym bilden. Das führt zum Modell bei dem α-und β-Untereinheiten eine heterotetrameren Struktur bilden, wie es schon zuvor im Gram-positiven Bakterium *Bacillus stearothermophilus* beschrieben worden ist.

In dieser Studie konnte erstmals gezeigt werden, dass die GlgC und GlgD Proteine von Milchsäurebakterien miteinander interagieren. Außerdem wurde in dieser Studie auch eine niedrige, ATP-abhängige enzymatische Aktivität der GlgD Protein in Abwesenheit von GlgC beobachtet. Die Fähigkeit des GlgD Proteins ADP-Glc zu produzieren deutet auf eine mögliche auch katalytische und regulatorische Funktion des Proteins hin. Die ADP-Glc-PPase Aktivität wird offenbar in bestimmten LAB durch einen Protein-Komplex gebildet, der durch die Gene *glgC* und *glgD* kodiert wird. Diese Gene sind in folgenden LAB-Stämmen konserviert: *Lactococcus lactis* subsp. *cremoris* MG1363 und *Lactobacillus plantarum* WCFS1. Darüber hinaus weisen erfolglose Versuche zur von *glgD* getrennten Expression von *glgC* auf die Wichtigkeit von GlgD für die stabile und

lösliche Expression von GlgC hin, der genaue Grund dafür ist unbekannt. Weitere Untersuchungen sind notwendig, um die vielen regulatorischen Aspekte des Glykogen Metabolismus in LAB sowohl auf Transkriptions- wie auch auf Translationsebene zu verstehen. Es könnte auch möglich sein, dass diese beiden Gene essentiell für das Wachstum dieser Arten sind, da sie trotz verschiedener Versuche mit verschiedenen Vektoren nicht deletiert werden konnten.

Eine weitere wichtige Beobachtung dieser Studie wies darauf hin, dass UTP als Substrat für das gereinigte GlgD ist, ein Anzeichen für eine alternative Reaktion zur Produktion von UDP-Glucose. Dies könnte ein Hinweis für einen alternativen Weg der Glykogen-Biosynthese sein. Die Beobachtung, dass die glgC- und glgD-Gene offenbar essentiell sind und dass es möglicherweise einen alternativen Weg für die Glykogen-Biosynthese gibt, deutet darauf hin, dass Glykogen eine wichtige Rolle für das Überleben dieser LAB-Stämme spielt.

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